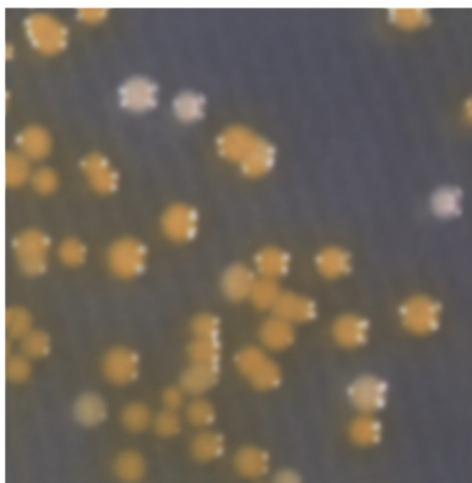

3



Pseudomonas

***Biosynthesis
of
Macromolecules
and
Molecular
Metabolism***



Edited by Juan-Luis Ramos

Pseudomonas

Pseudomonas

Edited by Juan-Luis Ramos, *CSIC, Granada, Spain*

Volume 1: Genomics, Life Style and Molecular Architecture

Volume 2: Virulence and Gene Regulation

Volume 3: Biosynthesis of Macromolecules and Molecular Metabolism

Pseudomonas

Volume 3 Biosynthesis of Macromolecules and Molecular Metabolism

Edited by

Juan-Luis Ramos

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Granada, Spain

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PREFACE

The time was ripe for a series of books on *Pseudomonas* to see the light. About 18 years have passed since Jack Sokatch first published his outstanding *The Biology of Pseudomonas* back in 1986. This was followed by two books published by the ASM that contained the presentations of the *Pseudomonas* meetings held in Chicago in 1989 and Trieste in 1991. The earlier volume was edited by Simon Silver, Al Chakrabarty, Barbara Iglewski and Sam Kaplan, and the later by Enrica Galli, Simon Silver and Bernard Witholt.

The present series of books was conceived at a meeting with Kluwer staff members in August 2002 during the XI IUMS conference in Paris. In less than a year a group of outstanding scientists in the field, after devoting much of their valuable time, managed to complete their manuscripts for the three volumes of the series. It has been an honor for me to work with them.

The review process has also been of great importance to ensure the high standard of each chapter. Renowned scientists have participated in the review, correction and editing of the chapters. Their assistance is immensely appreciated. I would like to express my most sincere appreciation to:

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Fernando Rojo	Masao Tsuda	

There has been growing interest in pseudomonads and a particular urge to understand the biology underlying the complex metabolism of these ubiquitous microbes. These bacteria are capable of colonizing a wide range of niches, including the soil, the plant rhizosphere and phyllosphere, and animal tissues; more recently they have attracted attention because of their capacity to form biofilms, a characteristic with potentially important medical and environmental implications.

There has been an explosion of vital new information about the genus *Pseudomonas*. A rapid search for articles containing the word “*Pseudomonas*” in the title in the last 10 years produces more than 6,000 articles! Consequently, we cannot cover all possible topics relevant to this genus in just three volumes, although our intention has been to be as thorough as possible. To organize the books, various topics have been grouped under a common heading, although this has some limitations since certain chapters may seem equally appropriate under different headings.

In Volume 1, the first chapter provides clues to the definition of the true *Pseudomonas* genus and gives a historical perspective of research to date. The insights in this chapter will undoubtedly help us to ascribe potential new isolates to this genus. Several recent advances in genomics, plasmids and phage biology, as well as a wealth of useful tools, are grouped under the heading “Genomics.” These chapters reveal the basis for diversity within the genus *Pseudomonas* at the molecular level, as well as key structural features. This section also describes a wide range of molecular tools to study gene expression and cloning in *Pseudomonas*, which have also been of great use in the analysis of other organisms. These chapters are therefore of potential interest to colleagues working in other genera in addition to *Pseudomonas*.

The section “Life styles” comprises a series of fascinating chapters that explain where pseudomonads can be found, their role in a particular niche, and how they interact with other members of the ecological community. Volume 1 concludes with a series of chapters on the architecture of *Pseudomonas* which explain the structure of the cell surfaces, their ability to

sense nutrients, and the use of the flagellar system of move toward chemoattractants or away from chemorepellent compounds. The molecular basis of cell architecture is critical to understand life styles, such as adhesion and secretion or extrusion of toxic substances including antibiotics and solvents. In all, I believe that the chapters included in this volume will by themselves constitute a book of general interest for microbiologists working on the biology of *Pseudomonas*.

Volume 2 deals with two important issues: virulence and gene regulation. The chapters under the heading "Virulence" deal with well-established virulence factors which make *Pseudomonas aeruginosa*, an opportunistic pathogen, and *Pseudomonas syringae*, a plant pathogen. Some issues on virulence were dealt with in Volume 1 under the heading "Life styles"; in Volume 2, the aim is to provide insights into the molecular mechanisms of virulence. In phytopathogens, their life style overlaps with some beneficial properties of non-pathogenic soil bacteria, but what actually makes them different is critical to our understanding of the biology of this group of *Pseudomonas*.

An astonishing finding from the analysis of the genomes sequenced to date is the wide battery of regulatory genes found in *Pseudomonas*. Also surprising is the wide range of alternative sigma factors encoded by these bacteria. Many of these factors belong to the ECF family and are involved in iron acquisition. The regulators of *Pseudomonas* can be placed into two broad groups, namely those that belong to the two-component class, in which a sensor protein "senses" the signal and triggers the actual regulator(s), and those of a second group in which sensing and regulatory functions are located in a single polypeptide. Volume 2 contains the most exhaustive and up-to-date compilation available of σ^{70} -dependent promoters in the genus *Pseudomonas*.

Volume 3 comprises the sections "Macromolecules," "Alternative respiratory substrates," "Catabolism and biotransformations" and "Secondary metabolism." The chapters in the section titled "Macromolecules" review some of the most complex metabolic pathways in the bacterial kingdom, and deal with the biosynthesis of LPS, fatty acids, alginate, rhamnolipids, cyclic lipopeptides, heme groups and vitamin B12.

Pseudomonads are well known for their extreme nutritional versatility and their ability to produce added-value products from simple, cheap carbon and nitrogen sources. The chapters in Volume 3 deal with the metabolism of certain amino acids and other natural compounds such as alkanes, as well as some xenobiotics and recalcitrant compounds such as aromatics. Some of the enzymatic properties of pseudomonads have been exploited to produce added-value products, which makes non-pathogenic *Pseudomonas* strains of great interest for certain industrial processes. This, in addition to the ability of certain strains to biosynthesize secondary products and influence the life style of certain strains, also makes them of interest for the industry.

In recent years, it has become clearer that pseudomonads are able to colonize anaerobic niches. In these niches many strains are able to respire alternative electron acceptors, and the process of denitrification is indeed well understood in some strains of the genus *Pseudomonas*. More recently, a strain able to respire nitroorganic compounds has been reported, and recent studies have revealed that this property may be more widespread than was initially thought.

There remains no doubt in my mind that in the next 10 years we will see myriad articles dealing with *Pseudomonas* and shedding further light on our current understanding of the broad group of bacteria in the genus *Pseudomonas* as it is now defined. However, I am confident that this series of books has assembled a significant part of the current knowledge of *Pseudomonas* in the best possible manner. More importantly, I hope it will open new lines of research that will lead to a better understanding of this group of saprophytic microorganisms.

Last but not least, I would like to acknowledge the enthusiasm and assistance of Carmen Lorente in the compilation of the three volumes that constitute the *Pseudomonas* series.

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Pseudomonas

MACROMOLECULES

LIPOPOLYSACCHARIDES OF *PSEUDOMONAS AERUGINOSA*

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1. INTRODUCTION

The lipopolysaccharide (LPS) of *Pseudomonas aeruginosa* is a major constituent of the outer leaflet of the outer membrane of the Gram-negative bacterial cell wall. Due to its surface location, LPS plays a critical role in the structural integrity of the outer membrane and in the interaction of the bacterium with its environment. In the human host, LPS shed by bacteria is usually bound by LPS-binding protein^{106, 178}, and is transferred to the CD14 receptor¹⁹⁸ on macrophages inducing the release of proinflammatory cytokines including tumor necrosis factor- α , interleukin 1 (IL-1), IL-6, IL-8 and IL-10¹¹⁵. In addition, it has been shown that LPS of bacteria interacts with Toll-like receptor 4 on host membranes and induces the release of cytokines¹⁴⁷. Properly regulated release of these inflammatory mediators is part of innate immunity against bacterial infections. However, excessive stimulation of the immune systems by LPS can result in septic shock and even death¹¹⁵. Therefore, a better understanding of the biosynthesis of *P. aeruginosa* LPS will provide the means to develop methods to block the interactions between LPS and host receptors.

P. aeruginosa LPS is composed of three distinct regions: (a) lipid A, which anchors the LPS molecule in the outer membrane and is covalently attached to the core, (b) core oligosaccharide (OS), which can be divided into

inner- and outer-core regions, and (c) the long chain polysaccharides (O antigen), which provides hydrophilic charges to the cell surface, and forms a surface layer that protects the bacterium from host defenses and hazardous environments. The majority of *P. aeruginosa* in natural environments and from clinical sources produce two chemically distinct forms of O antigens that are attached to the lipid A-core. On the basis of their resolution in liquid chromatography, LPS bearing these distinct forms of O antigens are called A-band and B-band LPS. In this chapter, we will provide an in-depth review on the current knowledge of LPS biosynthesis in *P. aeruginosa*, with particular focus on the genetic and biochemical evidence collected by our laboratory and other researchers.

2. LIPID A BIOSYNTHESIS IN

PSEUDOMONAS AERUGINOSA

2.1. Structure of Lipid A

The structure of lipid A in LPS of many different *P. aeruginosa* strains, including PAO1 and PAK, which are standard wild-type laboratory strains, has been determined by many groups^{20, 56, 87, 100}. The lipid A molecule consists of a β -1,6-linked D-glucosamine disaccharide [β -D-GlcN-(1 \rightarrow 6)-D-GlcN] that is phosphorylated in positions 4' and 1 with the attachment site of the core OS at position 6'. The diglucosamine backbone is substituted to varying degrees with fatty acid chains and as a result, hexaacyl, pentaacyl and tetraacyl species are produced. The major lipid-A species that is synthesized, however, is the pentaacyl lipid A form that contains two amide-bound 3-O-acylated (*R*)-3-hydroxydodecanoic acid groups [12:0(3-OH)] at positions 2 and 2' of the GlcN disaccharide and one ester-bound (*R*)-3-hydroxydecanoic acid group [10:0(3-OH)] at position 3' (Figure 1A). The 3-hydroxyl group of the two amide-linked 12:0(3-OH) residues are acylated by either dodecanoic (12:0) or (*S*)-2-hydroxydodecanoic acid [12:0(2-OH)]. This can lead to structural heterogeneity within the lipid A molecule, and consequently, *P. aeruginosa* has been found to synthesize the pentaacyl lipid A form with three acylation patterns. One form of lipid A is synthesized with the two amide-linked 12:0(3-OH) residues acylated by two 12:0 groups. The other forms contain 12:0(2-OH) at GlcN I and 12:0 at GlcN II or with the reverse distribution, while lipid A carrying two 12:0(2-OH) residues at both GlcN I and GlcN II do not appear to be synthesized.

Interestingly, *P. aeruginosa* is capable of synthesizing different forms of lipid A in response to environmental conditions that include magnesium-limiting growth medium and conditions encountered during different types of human infections. The predominant lipid A form produced by *P. aeruginosa* strain PAO1 and PAK when grown in high-magnesium conditions (1 mM)

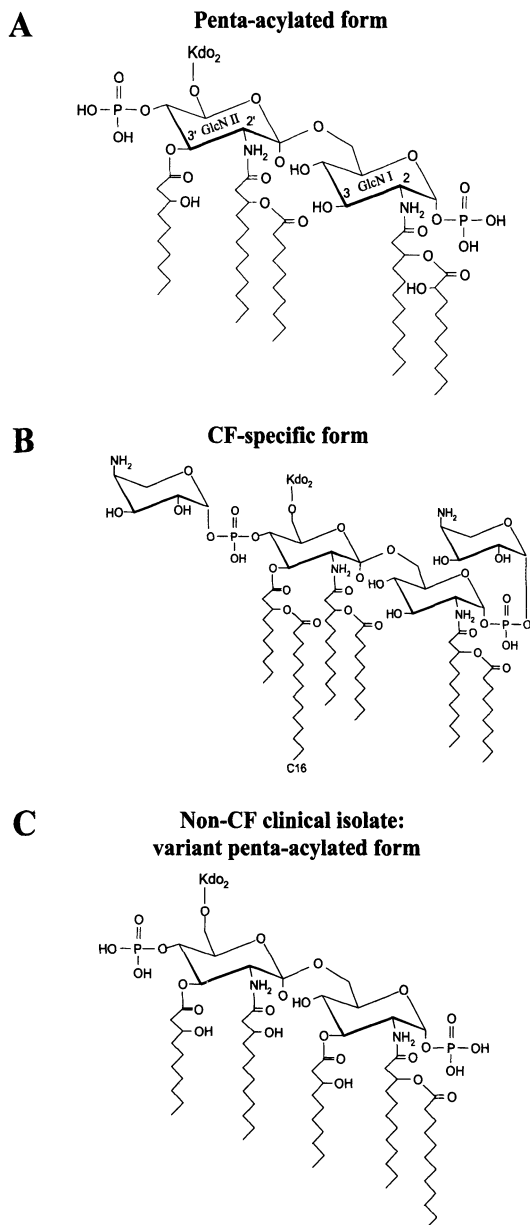


Figure 1. Various structures of *P. aeruginosa* lipid A. A. The predominant penta-acylated form of lipid A found in *P. aeruginosa* strains, including PAK and PAO1 when grown in high-magnesium conditions. B. Modified lipid A found in LPS from *P. aeruginosa* CF-clinical isolates containing 4-amino-4-deoxy-L-arabinose at the 1 and 4' phosphates and a palmitate at the 3-hydroxydecanoic acid, bound via an ester linkage to the 3' carbon. C. The non-CF dominant penta-acylated lipid A found in blood and bronchiectasis isolates when grown in Luria-Bertani media. C16 refers to the 16-carbon chain length of the palmitate substitution. The structures were illustrated based on data from refs [20], [56], [87], [100].

contains a penta-acylated moiety⁵⁶, a structure similar to those reported previously by other groups^{20, 87, 100}. The same penta-acylated form of lipid A was observed when PAK was grown in low-magnesium medium (8 μ M). However, PAK also produces three other forms of the penta-acylated lipid A. One penta-acylated form is modified by the addition of palmitate (hexadecanoic acid) on the 3-hydroxydecanoic acid, which is bound via an ester linkage to the 3' carbon. The other penta-acylated forms are characterized by the addition of palmitate as well as substitutions of 4-amino-4-deoxy-L-arabinose (aminoarabinose) to the 1 or 4' phosphates (or both). Using MALDI-TOF mass spectrometry to examine the structure of lipid A from *P. aeruginosa* isolated from cystic fibrosis (CF) patients, Ernst *et al.*⁵⁶ identified several penta-acylated forms of the molecule. One of these was similar to the one previously described for strain PAK growing in high-magnesium conditions. Another has palmitate modification and the third form has palmitate modification as well as aminoarabinose substitutions at the 1 and 4' phosphate positions (Figure 1B). The highest level of lipid A modification with palmitate observed is 33% in one of the clinical isolates. It was intriguing that the palmitate and aminoarabinose modifications were lost when this CF isolate was continuously passaged in Luria-Bertani broth medium⁵⁶.

Different structures of lipid A are synthesized in non-CF clinical isolates⁵⁶. The dominant penta-acylated lipid A form isolated from minimally passaged non-CF strains (isolated from sepsis and bronchiectasis) was markedly different in the fatty acid distribution than the dominant penta-acylated lipid A species isolated from PAK, PAO1 and the CF isolates (Figure 1C). Firstly, the dominant penta-acylated form of lipid A in the non-CF isolates contains 3-OH-C10:0 at the 3 position whereas the lipid A from the CF isolates and PAK did not have this fatty acyl chain. Secondly, the non-CF lipid A lacked the 2-OH-C12:0 at the 2' position, and thirdly, the addition of palmitate and aminoarabinose to this penta-acylated lipid A form was not observed in any of the non-CF clinical isolates even when grown in magnesium-limiting conditions. However, when these non-CF isolates were grown in magnesium-limiting media, they produced the structurally distinct CF-specific penta-acylated lipid A forms modified with palmitate and aminoarabinose. Therefore, these studies demonstrate that *P. aeruginosa* has the remarkable ability to synthesize a variety of lipid A structures in vivo in response to changes in its environment.

2.2. The Genes of Lipid A Biosynthesis

In *P. aeruginosa*, many genes that are highly homologous to those involved in *Escherichia coli* lipid A biosynthesis have been identified. The gene products of *lpxA* and *lpxC* are required for the first two and most conserved steps and have been characterized at the biochemical level. The other

genes thought to be involved in the remaining steps of lipid A biosynthesis in *P. aeruginosa* have been annotated and assigned putative functions based on homology of their encoded proteins with enzymes of *E. coli* lipid A biosynthesis (*Pseudomonas aeruginosa* Community Annotation Project-PseudoCAP [http://www.pseudomonas.com/AnnotationListByFunction.asp?Function=Cell%20wall%20/%20LPS%20/%20capsule]; The *Pseudomonas* Genome Project¹⁷⁶). Variations in the structure of *P. aeruginosa* lipid A in comparison to that of *E. coli* include differences in acyl chain length and distribution. These structural differences may account for the presence of unique enzymes in *P. aeruginosa* that have not yet been identified because they do not share significant homology with other characterized proteins. The molecular genetics and biochemistry of lipid A biosynthesis are best characterized in *E. coli* and have been thoroughly reviewed by Raetz and Whitfield¹⁵⁰. Therefore, only a brief description of the enzymes identified in the pathway for *P. aeruginosa* lipid A biosynthesis will be discussed here.

The first step of lipid A synthesis in *P. aeruginosa* is the acylation of UDP-*N*-acetylglucosamine (UDP-GlcNAc) at the C-3 position with a 10-carbon acyl chain by LpxA, a UDP-*N*-acetylglucosamine-3-*O*-acyltransferase⁵³. The product of this reaction, UDP-3-*O*-(*R*-3-hydroxydecanoyl)-*N*-acetylglucosamine, is deacetylated at the C-2 position to yield a free amino group by the enzyme, LpxC, a UDP-3-*O*-acyl-GlcNAc deacetylase⁷⁸. PA3646, a homologue of *E. coli* LpxD, is a putative acyltransferase that transfers hydroxydodecanoic acid to UDP-3-hydroxydecanoyl-glucosamine to produce UDP-2-hydroxydodecanoyl-3-hydroxydecanoyl-GlcN⁸⁹. In *E. coli*, UDP-2,3-diacylglucosamine is cleaved at its phosphate bond by a pyrophosphatase called LpxH, to form 2,3-diacylglucosamine-1-phosphate or what is also called lipid X⁸. At present, an LpxH homologue has not been identified in the *P. aeruginosa* genome. A condensation reaction between UDP-2,3-diacylglucosamine and lipid X generates a β , 1'-6 linked disaccharide and this reaction requires the activity of the disaccharide synthase LpxB, encoded putatively by PA3643¹⁴⁸. Phosphorylation at the 4' position of the disaccharide to form lipid IV_A is presumably catalyzed by a specific kinase⁶¹ and the likely candidate is the protein encoded by PA2981, an *E. coli* LpxK homologue. The final steps in the biosynthesis of lipid A differ between enteric and non-enteric bacteria. In enteric bacteria, once lipid IV_A is made, the addition of two 3-deoxy-D-manno-octulosonic acid residues to lipid IV_A precedes the addition of the fifth and sixth acyl chains²⁴. In *P. aeruginosa*, the lipid IV_A is first acylated with two dodecanoic acid chains, which are *O*-linked to the acyl chains at the 2 and 2' positions before the addition of the 3-deoxy-D-manno-octulosonic acid residues^{66, 128}. Proteins encoded by PA0011 and PA3242 of the *P. aeruginosa* genome have been annotated to have the function of lauroyltransferases that add these additional acyl groups and these putative proteins are homologues of *E. coli* LpxL³².

A *phoP/phoQ* two-component regulatory system in *P. aeruginosa* described by Macfarlane *et al.*¹¹⁶ has been implicated in the regulation of genes required for the addition of aminoarabinose and palmitate to lipid A in magnesium-starvation conditions. PhoQ is a transmembrane histidine kinase that responds to extracellular concentrations of divalent cations, such as magnesium and calcium. In conditions of magnesium starvation, PhoQ activates the PhoP-response regulator, which controls the expression of many genes in this type of environment⁶⁹. Negative-ion MALDI-TOF mass spectroscopy analysis showed that the lipid A from wild-type *P. aeruginosa* strain PAK grown in low-magnesium conditions contains aminoarabinose and palmitate whereas the lipid A from a *phoP* null mutant does not⁵⁶. At present, the genes that are presumably turned on by the PhoP/PhoQ system and mediate the transfer of palmitate to lipid A in *P. aeruginosa* have not been identified. The aminoarabinose substitution of *P. aeruginosa* lipid A is thought to be mediated by PA3556 (PseudoCAP annotation), which encodes a protein with sequence similarity to *E. coli* ArnT, an inner membrane enzyme that adds two units of aminoarabinose to lipid A precursors containing a 3-deoxy-D-manno-octulosonic acid disaccharide¹⁷⁹. Studies comparing the biophysical properties of the PhoQ sensors from *P. aeruginosa* and *E. coli* show that the PhoQ proteins from these two organisms differ in their structural response to divalent cations. PhoQ from *P. aeruginosa* may recognize additional signals or respond differently than *E. coli* and *Salmonella enterica* PhoQ in certain environments¹⁰⁹. Furthermore, the distinct mechanisms of signal detection between *P. aeruginosa* and *E. coli* suggest that lipid A modifications in these two organisms may be regulated differently.

The first steps of lipid A biosynthesis in Gram-negative bacteria are essential for viability and enzymes required for these steps have been identified as potential candidates for the development of antimicrobial targets^{65, 71, 137}. Consequently, *P. aeruginosa* LpxA and LpxC have been characterized at the genetic and biochemical level. *P. aeruginosa* LpxA catalyzes the acylation of UDP-GlcNAc at the C-3 position with a 10-carbon acyl chain and is specific for a hydroxydecanoyl-acyl carrier protein (3-OH-C10-ACP)^{53, 195}. *P. aeruginosa* LpxA is 54% identical to *E. coli* LpxA, which is specific for the 14-carbon acyl chain of 3-hydroxymyristoyl-acyl carrier protein (3-OH-C14-ACP)⁷. When *P. aeruginosa* LpxA was overexpressed and the purified protein assayed in *E. coli*, the enzyme showed a greater than 1,000-fold preference for 3-OH-C10-ACP over 3-OH-C14-ACP^{53, 199}. The hydrocarbon chain length preference of *P. aeruginosa* LpxA was further demonstrated *in vivo*, as expression of *P. aeruginosa* *lpxA* in a *Neisseria meningitidis* *lpxA* mutant resulted in the production of hybrid lipid A molecules with 3-OH-C10 as the major fatty acid¹⁷⁵. LpxA enzymes are very selective for the hydrocarbon length of the acyl carrier protein, but the mechanism of this selectivity is not well

understood. To facilitate our understanding of the exquisite hydrocarbon ruler in LpxA proteins, the X-ray crystal structure of *E. coli* LpxA has been solved at a 2.6-Å resolution and a model of the native *E. coli* LpxA homotrimer has been resolved¹⁴⁹. A feature of particular interest is a novel left-handed parallel β -helix that appears to be directed by 24 hexapeptide repeats that are found in the N-terminal two thirds of each monomer. The *P. aeruginosa* enzyme contains hexapeptide repeat motifs with nearly identical spacings to that of the *E. coli* LpxA and therefore these proteins are predicted to have similar overall folds⁵³. A significant discovery was the identification of a single amino acid within the putative catalytic site that is a key determinant of acyl chain selectivity. The wild-type *P. aeruginosa* LpxA prefers a 3-OH-C10-ACP and a switch from methionine to glycine at amino acid 169 results in a protein that prefers a 3-OH-C14-ACP. The wild-type *E. coli* LpxA is selective for 3-OH-C14-ACP and a reciprocal amino acid switch from glycine to methionine in a similar position resulted in a 3-OH-C10-ACP-dependent enzyme, which catalyzed the synthesis of the expected hybrid lipid A species¹⁹⁹. These data suggest that LpxA has a precise mechanism for chain length selectivity. A detailed understanding of the function of this enzyme may assist in the preparation of lipid A analogues which have the potential for therapeutic purposes that are presently synthesized using sophisticated chemical processes²⁵.

The second step in lipid A biosynthesis is the *N*-deacetylation of UDP-3-*O*-(*R*-3-hydroxydecanoyl)-*N*-acetylglucosamine by LpxC, a UDP-3-*O*-acyl-GlcNAc deacetylase. This is proposed to be the committing reaction step of the lipid A pathway^{172, 204}. *P. aeruginosa* LpxC has been purified to homogeneity and has high deacetylase activity when assayed in vitro using UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN[³H]Ac as a substrate⁷⁸. Presumably, in vivo, *P. aeruginosa* LpxC recognizes UDP-3-*O*-(*R*-3-hydroxydecanoyl)-GlcNAc. Although the activity of purified *P. aeruginosa* LpxC with its native substrate, UDP-3-*O*-(*R*-3-hydroxydecanoyl)-GlcNAc, has not been tested, LpxC protein prepared from wild-type crude extracts of *P. aeruginosa* was shown to be relatively non-specific with respect to the substrate's acyl chain length¹⁹⁵.

LpxC is an excellent target for the design of Gram-negative specific antimicrobial drugs. The *lpxC* gene is essential for viability¹⁵ and LpxC has been identified in more than 40 Gram-negative species. LpxC is a zinc-containing enzyme that requires zinc for its catalytic activity⁸¹. Data from sequence alignments, mutagenesis studies and extended X-ray absorption fine structure spectroscopy indicate a novel zinc binding motif in LpxC proteins that is not found in any other metalloproteins studied to date^{80, 124}. Compounds containing hydroxamic acids are thought to inhibit LpxC activity by chelating the zinc ion. A family of hydroxamic acids has been found to inhibit the activity of purified *E. coli* LpxC in vitro and growth of the bacterium in animal models^{31, 137, 143, 144}. However, this specific class of inhibitors has no effect on

the growth of *P. aeruginosa*. Several novel compounds that include a carbohydrate-derived hydroxamic acid called compound 8 have been shown to inhibit the activity of purified *P. aeruginosa* LpxC^{79, 90, 110} and the effect of these inhibitors against *P. aeruginosa* and other Gram-negative organisms is currently being evaluated.

3. STRUCTURE AND BIOSYNTHESIS OF CORE OLIGOSACCHARIDE

3.1. Structure of the Core Oligosaccharide

P. aeruginosa produces two distinct core OS glycoforms. One of these core glycoforms is the acceptor molecule for the covalent attachment of the long chain A- or B-band PS while the other core form remains uncapped (Figure 2). The two core glycoforms differ in the outer-core region whereas the inner core remains conserved. The inner core is composed of two residues of 3-deoxy-D-manno-octulosonic acid (Kdo) and two residues of L-glycero-D-manno-heptose (L,D-Hep). The inner core also contains a unique O-carbamoyl substitution at C-7 of the L,D-Hep residue located proximal to the outer core¹⁶. The substitution of a sugar by a carbamoyl residue is unique in bacterial LPS and the only other report of a carbamoylated sugar is the terminal N-acylated and N-methylated GlcN residue of the Nod factor produced by *Azorhizobium caulinodans*¹²⁷. 7-O-carbamoyl-L,D-Hep has also been identified in the LPS of other *P. aeruginosa* strains including rough mutants (PAC605, PAC557, PAC608, and R5), wild-type strains (Fisher immunotypes 1, 2 and 7), and other *Pseudomonas* species including *P. fluorescens*, *P. syringae* and *P. wieringae*¹⁶. Consequently, the presence of the carbamoyl substituent could serve as a diagnostic marker for *Pseudomonas* spp. of the RNA group I described by Palleroni¹³⁹.

One distinguishing feature of the *P. aeruginosa* inner core is the remarkably high degree of phosphorylation associated with the heptose region. Determination of the phosphorylation patterns in this region has proven to be difficult and early studies have reported as many as 10 phosphate groups associated with an LPS molecule^{134, 193, 194}. The exact phosphorylation sites were not identified until an accurate core OS structure of a mutant derived from strain PAO1 was described by Masoud *et al.*¹²⁰. The location of two phosphate groups was found to occur at C-2 and C-4 of L,D-HepI (HepI being the first Hep residue linked to lipid A). These phosphates are present in stoichiometric amounts and similar results have been reported in numerous studies^{121, 163–165} on other *P. aeruginosa* strains. A third phosphorylation substitution has been found at C-6 of either L,D-HepI¹⁶³ or L,D-HepII¹⁶⁵. In a recent study by

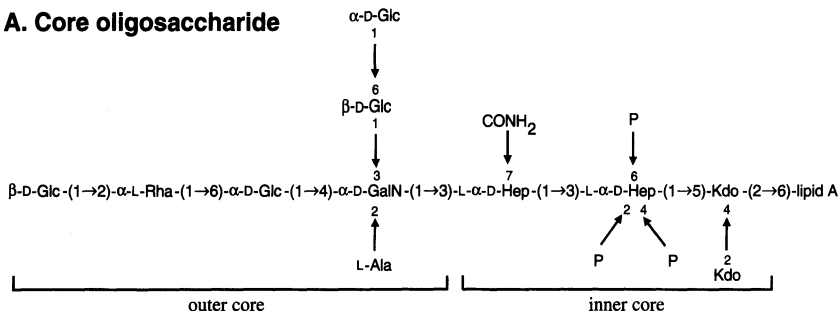
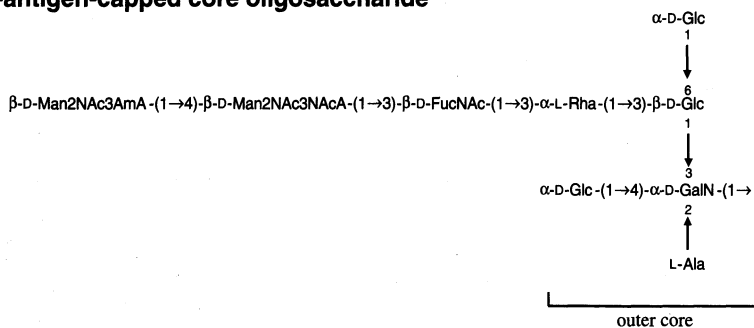
A. Core oligosaccharide**B. O-antigen-capped core oligosaccharide**

Figure 2. Structures of the core OS from *P. aeruginosa* strain PAO1. Two distinct core OS have been elucidated. A. One of them is devoid of O antigen and is referred to as “uncapped” core OS. Note the L-rhamnose is in a α -1,6 linkage to an α -D-glucose in the main chain. B. The other core OS contains O antigen linked to L-rhamnose in an α -1,3 linkage to β -D-glucose in the branch chain. Glc, glucose; Rha, rhamnose; GalN, galactosamine; Ala, alanine; CONH₂, carbamoyl group; P, phosphate; Hep, L-glycero-D-manno-heptose; Kdo, 3-deoxy-D-manno-octulosonic acid. The structures were drawn based on data from refs [163], [164].

Knirel *et al.*⁹⁵, four major phosphorylation sites were identified on the LPS of a CF clinical isolate and these phosphates were found at C-2 and C-4 of HepI and C-4 and C-6 of HepII.

The LPS outer-core region contains four D-glucose (D-Glc), one L-rhamnose (L-Rha) and one N-(L-alanyl)-D-galactosamine. The outer core occurs as two isomeric glycoforms differing in the position of L-Rha and one D-Glc residue. Although both core types contain an L-Rha residue linked to D-Glc, the uncapped core glycoform contains L-Rha in an α -1,6 linkage to an α -D-Glc in the main chain, whereas the second core glycoform capped with O antigen contains L-Rha in an α -1,3 linkage to β -D-Glc in the branch chain. This α -1,3 linked L-Rha is the attachment point for the O antigen in strain PAO1 (serotype O5)¹⁶⁴. In a study by Bystrova *et al.*³⁰, they observed

two core glycoforms in *P. aeruginosa* strain 170041, classified as immunotype 1 or serotype O6. The chemical structure of the core OS and linkage of the L-Rha of the core capped in the LPS of this strain is the same as that reported previously by Sadovskaya *et al.*¹⁶⁴ for PAO1 (serotype O5). The only difference between the uncapped core OS of strain PAO1 and 170041 is that the core of 170041 lacks the terminal D-Glc residue. The terminal D-Glc found in the outer core of PAO1 (serotype O5) was also shown to be absent in the outer core of strain PAK (which also produces serotype O6 O antigen)¹²¹ and is consistent with the structural data presented for strain 170041, immunotype 1 (serotype O6). This structural distinction between the outer core OS of serotypes O5 and O6 was substantiated by immunochemical data whereby an outer core-specific monoclonal antibody (mAb) 101 reacted to a core LPS band of O5 but not O6 in Western immunoblots⁴³.

In a recent study, Knirel *et al.*⁹⁵ investigated the structure of the core LPS of a rough, CF clinical isolate. It is important to note that LPS from chronic CF clinical isolates of *P. aeruginosa* is usually devoid of O-antigen PS^{59, 72, 103, 141}. These researchers noted that CF clinical isolate 2192 synthesizes two different core glycoforms even in the absence of the O-antigen PS⁹⁵ and the presence of two distinct core forms is a characteristic similar to that observed in the wild-type strain PAO1. However, it is of interest to note that the core glycoform required for attachment of the O antigen was produced despite the absence of capping by O-PS in the LPS preparation from the clinical isolate. Comparison of the two LPS core glycoforms between isolate 2192 and strain PAO1 revealed that the core glycoform available for O attachment has the same structure; however, the uncapped core from the CF isolate lacks the terminal β -D-Glc residue attached at C-2 of the L-Rha of the main chain that is found in PAO1. A new finding in the core OS of clinical isolate 2192 is the O-acetylation that occurs at four sites, although the location of only one O-acetylation site was found at the L-Rha residue⁹⁵. To date, O-acetylation of core LPS has been found in other bacteria including *P. fluorescens*⁹⁶ with the only other report of this modification in the *P. aeruginosa* species in strain 170041³⁰. It is apparent that O-acetylation of core OS is nonstoichiometric, and at present, the exact positions of the O-acetyl groups and the role of O-acetylation in the LPS core are unknown.

The elucidation of the complete structures of the two distinct core glycoforms has a profound impact with respect to our understanding of core biosynthesis. Previously, many groups reported partial structures. Alternatively, by using LPS mutants, elucidation of complete structures of truncated core OS for *P. aeruginosa* serogroups O2, O3, O5 and O6 were attained^{154, 120, 121, 162, 163, 165}. The study by Sadovskaya *et al.*¹⁶⁴ was the first to have accomplished the elucidation of the chemical structure of the outer-core region in a fully assembled O-chain containing LPS. The knowledge of the

existence of two core OS types facilitated a better understanding of the different structural data obtained from LPS mutants, and more importantly, it allowed us to pursue functional characterization of the genes involved in biosynthesis and assembly of the LPS core using genetic and biochemical approaches.

3.2. Genetic Organization of Core Oligosaccharide Biosynthesis Genes

Earlier studies by our group showed that an operon consisting of *waaFwaaCwapGwaaPwapPwapQ* is associated with the production of *P. aeruginosa* core OS^{42, 123, 187}. By using a similarity search program called Basic Local Alignment Search Tool (BLAST); National Center for Biotechnology Information [<http://www.ncbi.nlm.nih.gov/>], we compared the nucleotide sequence of the operon to the whole genome sequence of *P. aeruginosa* PAO1 and found that these genes are part of a larger gene cluster. The PAO1 core OS gene cluster (Figure 3) appears to contain four independently regulated operons that span the region corresponding to PA5012-PA4996 of the PAO1 genome (PseudoCAP [<http://www.cmdr.ubc.ca/bobh/PAAP.html>]; The *Pseudomonas* Genome Project¹⁷⁶). At the 5' end of the core cluster, *waaF* (PA5012) is the first gene of the major operon that contains 12 contiguous open reading frames (ORFs), which include *waaFwaaCwapGwaaPwapPwapQ*. These first six genes are involved in inner-core biosynthesis.

How the last six ORFs of the *waaF* operon contribute to core OS biosynthesis is not well understood. The ORF after *wapQ*, PA5006, encodes a protein

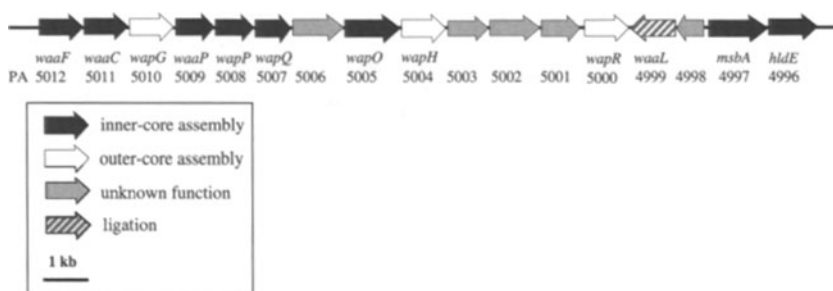


Figure 3. The genetic organization of the core OS biosynthetic gene cluster of *P. aeruginosa* strain PAO1. The predicted genes are designated by their ORF numbers (e.g., PA5012-PA4996), which has been assigned based on analysis of the PAO1 genome by Stover *et al.*¹⁷⁶. Those proteins whose function has been characterized experimentally or have significant homology to known proteins in the database have been assigned a gene name, for example, *waaF*, *waaC*, *wapG*, *waaP*, *wapP*, *wapQ*, *wapR*, *waaL*, *msbA* and *hldE*. The function of the gene products of this core cluster are described in detail in Sections 3.2–3.4. Scale bar equals 1 kb.

with some kinase-like features and it is hypothesized to be involved in phosphorylation of the inner-core region. Investigations in our laboratory and annotation information (PseudoCAP) have revealed that PA5005 and PA5004 may encode a putative carbamoyltransferase and glycosyltransferase, respectively. The next ORF, PA5003, is annotated (PseudoCAP) to encode a protein that is 48% similar to the protein encoded by *mig-14* of *Salmonella typhimurium*, which may be a putative transcriptional activator¹⁸². The *mig-14* gene is a host-induced virulence gene under the control of the PhoP/PhoQ two-component system¹⁸². This gene is required for fatal infection in a model of enteric fever¹⁸¹ and plays a role in bacterial resistance to antimicrobial peptides²³. Attempts in our laboratory to create a null mutation in PA5003 in *P. aeruginosa* PAO1 have been unsuccessful, implicating that its gene product is essential for viability in *P. aeruginosa*. This result is in contrast to those obtained for *S. typhimurium*, where a *mig-14* mutant has been generated²³. The mechanism by which *mig-14* contributes to pathogenicity in *S. enterica* is not yet known, therefore, it would be difficult to speculate the function of PA5003 in *P. aeruginosa* LPS synthesis. The last two ORFs of the *waaF* operon, PA5002 and PA5001, have no significant homology to any known proteins. Therefore, it remains to be determined as to what role these ORFs play in LPS-core biosynthesis.

Located at 158 bp downstream of the last ORF of the *waaF* operon is a single gene, *wapR* (PA5000), which encodes a rhamnosyltransferase involved in outer-core assembly¹⁴⁶. This gene is followed by two divergently transcribed operons, each of which contains two genes. The two-gene operon closest to *wapR* contains ORFs PA4998 and PA4999. Although PA4998 has no proposed function, PA4999 most likely encodes WaaL, the O-antigen ligase. Using a prediction of transmembrane helices in proteins program (TMHMM version 2.0; Center for Biological Sequence Analysis, The Technical University of Denmark [<http://www.cbs.dtu.dk/services/TMHMM-2.0/>]; refs [99], [171]), the protein encoded by PA4999 has 11 membrane spanning domains and its hydropathy profile is similar to those obtained from known WaaL proteins from *E. coli* and *S. enterica*⁷⁵. The second two-gene operon contains two ORFs (PA4997 and PA4996), which encode proteins similar to *E. coli msbA* and *hldE*. *E. coli* MsbA has been shown to be an essential ABC transporter for LPS^{51, 52, 208} while *hldE* encodes a bifunctional D- β -D-heptose-7-phosphate kinase/D- β -D-heptose-1-phosphate adenylyltransferase required for synthesis of ADP-L,D-Hep, the activated nucleotide donor for heptose residues for the inner core^{92, 183}.

Regulation of transcription elongation of PS gene clusters in many different organisms is controlled by RfaH and the *ops* element (operon polarity suppressor)¹⁰. RfaH is a processive elongation factor and the *ops* element is the nucleic acid recognition site for RfaH⁹. It is hypothesized that this system modifies the RNA polymerase complex to increase its processivity

and allows transcription to proceed over long distances. The *ops* element is a single 8-bp motif, 5'-ggcggtag-3', which has been found upstream of polysaccharide gene clusters from *Escherichia coli*, *Salmonella* spp., *Shigella flexneri*, *Yersinia enterocolitica*, *Vibrio cholerae* and *Klebsiella pneumoniae*¹⁰, as well as in the RP4 fertility operon of *P. aeruginosa*¹³³. In the *P. aeruginosa* *waaF* operon of 12 genes, an *ops* element seems very likely; however, an *ops* consensus sequence could not be located upstream of the *waaF* operon.

3.3. Enzymes for Inner-Core Oligosaccharide Synthesis

In *P. aeruginosa* strain PAO1, an operon containing three genes, *pyrG*, *kdsA* and *eno* is involved in the biosynthesis of the Kdo residue, which is the first sugar of the inner core¹⁸⁶. One of the steps in Kdo synthesis involves the condensation of phosphoenolpyruvate and arabinose-5-P to form Kdo-8-P and this reaction is catalyzed by a Kdo-8-P synthase called KdsA¹⁵⁸. Kdo-8-P is activated to cytidine monophosphate Kdo (CMP-Kdo) with cytidine triphosphate (CTP) as the nucleotide donor and CMP-Kdo serves as the activated sugar donor for transfer of Kdo residues to lipid A by the putative KDO transferase, encoded by PA4988 (PseudoCAP). PyrG is a CTP synthetase, which catalyzes the transfer of ammonia to UTP to form CTP. Eno is involved in the formation of phosphoenolpyruvate. *P. aeruginosa* PyrG, KdsA and Eno are 69%, 69% and 77% identical to PyrG, KdsA and Eno of *E. coli*, respectively. *P. aeruginosa* *kdsA* was shown to rescue a 42°C temperature-sensitive *S. typhimurium* *kdsA* mutant for growth at 42°C. The Kdo-8-P synthase activity of *E. coli* carrying *P. aeruginosa* *kdsA* on a plasmid was almost 6-fold higher than that of a control *E. coli* strain carrying a vector with no gene¹⁸⁶. *P. aeruginosa* *pyrG* could complement the growth of an *E. coli* *pyrG* mutant that is auxotrophic for cytidine. The *pyrG* operon is located outside the *waaF* gene cluster and a sigma-70 like promoter upstream of *pyrG* initiates transcription of the genes in this operon¹⁸⁶.

The first two genes of the *waaF* operon, *waaF* and *waaC*, encode heptosyltransferases I and II that are required for assembly of the heptose region of the inner core⁴². WapG has homology to *E. coli* WaaG, which is a UDP-glucose (heptosyl) LPS α -1,3-glucosyltransferase, that transfers D-Glc from UDP-D-Glc to HepII of the inner core²⁰². The first sugar of the outer core in *E. coli* is D-Glc. In contrast, the first outer-core residue in *P. aeruginosa* is N-(L-alanyl)-D-galactosamine. The homology between WaaG and WapG may be based partially on a common α -1,3 linkage of the first hexose residue to the acceptor molecule, HepII-inner core. We hypothesize that WapG transfers D-GalNAc from UDP-D-GalNAc to L,D-HepII of the inner core. Subsequently, D-GalNAc is deacetylated, followed by the addition of L-alanine, as by yet,

unidentified enzymes. Although many different strategies were used, a *wapG* mutant cannot be generated. *P. aeruginosa wapG* is unable to complement the LPS defect in an *E. coli waaG* mutant, demonstrating that despite the sequence similarity between WapG and WaaG at the protein level, these enzymes likely have different glycosyltransferase activities¹²³.

The heptose region of the inner core in *P. aeruginosa* has been shown to be substituted with up to four phosphate moieties and so far, three genes, *waaP*, *wapP* and *wapQ* have been implicated in phosphorylation¹⁸⁷. Amino acid sequence analysis shows WaaP, WapP and WapQ to contain consensus sequences of kinase-2 motifs. By performing nuclear magnetic resonance (NMR) and methylation linkage analysis, our group has demonstrated that WaaP is responsible for the addition of one phosphate to O4 of HepI¹⁸⁷. The WaaP protein has been purified and the detailed kinetics of its heptose kinase activity were measured using an enzyme-linked immunosorbent assay (ELISA) developed in our laboratory^{206, 207}. BLAST searches revealed that *P. aeruginosa* WaaP shares homology with eukaryotic-type protein kinases that belong to the serine/threonine kinase and the tyrosine kinase family. WaaP has significant identity in the conserved, functional residues of the protein kinases, which include, for example, the amino acid residues that are thought to interact with the phosphate groups of ATP, the phosphate donor. The hypothesis that WaaP could also be a protein kinase in addition to a sugar kinase was substantiated by its ability to phosphorylate an exogenous tyrosine-containing substrate²⁰⁶. In addition, purified WaaP was found to exhibit auto-phosphorylation activities when assayed in a self-phosphorylation chemiluminescence-based ELISA using an anti-phosphotyrosine antibody. MALDI-TOF mass spectrometry and proteolytic peptide mapping studies of the full-length purified WaaP indicate that eight tyrosine residues are phosphorylated. The hypothesis that WaaP utilizes a catalytic mechanism similar to that of eukaryotic type protein kinases was validated by site-directed mutagenesis of key catalytic residues and subsequent complementation assays. Therefore, from the results of detailed biochemical characterization studies, we conclude that *P. aeruginosa* WaaP is capable of three distinct activities, sugar kinase activity, protein kinase activity and self-phosphorylating tyrosine kinase activity²⁰⁶. *P. aeruginosa* WaaP also has homology to *E. coli* WaaP. Although both proteins have heptose kinase activity, these enzymes appear to be structurally different^{203, 206, 207}. Also, the *E. coli* WaaP protein does not contain the typical pattern of conserved domains that are characteristic of tyrosine kinases and no data has been reported to demonstrate protein tyrosine kinase activity for *E. coli* WaaP. Altogether, the additional protein kinase and auto-phosphorylating activities that *P. aeruginosa* WaaP has, in addition to the lethality of the *waaP* mutation in *P. aeruginosa* suggest that the mechanism by which the

inner-core heptose is phosphorylated in *P. aeruginosa* may be different than that of *E. coli* or that *P. aeruginosa* WaaP may have another role in core assembly. Experiments to crystallize *P. aeruginosa* WaaP is currently underway in our laboratory to help solve the mechanisms of the kinase activities.

The functions of *P. aeruginosa* WaaC, WaaF, WaaP and WapP in inner-core assembly was demonstrated by cross complementation of *S. typhimurium* *waaC*, *waaF* and *waaP* mutants, respectively, rather than by generating and characterizing defined *P. aeruginosa* mutants in those respective genes. The rationale of using this approach is due to the fact that repeated attempts to create null mutations in *P. aeruginosa* *waaC*, *waaF*, *wapG*, *waaP* and *wapP* were unsuccessful^{42, 123, 187}. A chromosomal *P. aeruginosa* *waaP* mutant was made only when the mutant was concomitantly carrying a copy of *waaP* in *trans*¹⁸⁷. In addition, despite numerous attempts by various research groups using different mutagenesis approaches, *P. aeruginosa* LPS mutants lacking inner core L,D-Hep or phosphate have never been isolated. These observations suggest that L,D-Hep-linked phosphate is essential for viability. The inability to generate a *P. aeruginosa* *wapG* mutant is consistent with this hypothesis and is further substantiated by comparing to the phenotype of an *E. coli* *waaG* mutant. The LPS from an *E. coli* *waaG* mutant is not only truncated after the inner-core heptose residues, but there is also an 80% total reduction in heptose phosphorylation²⁰². In *E. coli*, the addition of the first glucose residue of the outer core is required for complete phosphorylation of the inner core and is likely the reason why a *P. aeruginosa* *wapG* mutant cannot be constructed.

The observation of lethal consequences in *P. aeruginosa* inner-core mutants is in contrast to *E. coli* and *Salmonella*, where LPS mutants that lack the heptose region of the inner core are viable, though they exhibit a “deep-rough” phenotype. Some of the characteristics of this phenotype include changes in surface hydrophilicity and hypersensitivity to hydrophobic antibiotics, dyes and detergents^{135, 168}. The phosphate moiety likely participates in ionic interactions with divalent cations thereby causing cross-linking between the LPS molecules and serves to stabilize the outer membrane. Cross-linking LPS molecules may be especially important in *P. aeruginosa* since this bacterium is highly susceptible to lysis by agents that chelate divalent cations, such as ethylenediamine tetra-acetic acid^{55, 68}. Thus, attempts to perform detailed characterization of the inner core genes of *P. aeruginosa* has been hampered by the inability to create LPS null mutants. These mutants would be extremely important for two reasons. First, the phenotype of the mutant equivocally shows direct involvement of the gene product in LPS biosynthesis. Second, LPS isolated from the mutant provides the specific substrate molecules for in vitro assays and consequently, facilitates biochemical characterization.

3.4. Genes for Outer-Core Oligosaccharide Biosynthesis

The major sugar residues of the outer-core OSs are D-Glc and L-Rha and the nucleotide-activated sugar donors for addition of these sugars by glycosyltransferases to the inner core are UDP-D-Glc and dTDP-L-Rha. AlgC is a key enzyme in polysaccharide synthesis in *P. aeruginosa* because it is required for the synthesis of both UDP-D-Glc and dTDP-L-Rha and these nucleotide donors provide sugar residues not only for the assembly of the core OS^{34, 62}, but also for O-antigen PS⁶⁴ and rhamnolipids¹³⁶. The *algC* gene encodes a bifunctional enzyme that has both phosphoglucomutase (PGM) and phosphomannomutase (PMM) activity. The PGM activity catalyzes the conversion of glucose-6-phosphate to glucose-1-phosphate (Glc-1-P), which is a common intermediate between the biosynthetic pathways for UDP-D-Glc and dTDP-L-Rha. The conversion of Glc-1-P to UDP-D-Glc is catalyzed by GalU, a UDP-D-glucose pyrophosphorylase, while the conversion of Glc-1-P to dTDP-L-Rha requires numerous reaction steps catalyzed by the *rmlBDAC* operon. Synthesis of dTDP-L-Rha in bacteria has been characterized at the biochemical level^{62, 67} and the three-dimensional structure of RmlA from *P. aeruginosa* as well as the enzymatic mechanism of this protein has been solved^{21, 22}. Mutations in *rmlC* and *galU* in *P. aeruginosa* have been shown to abrogate the production of complete core OS^{47, 151}. The second enzyme activity of AlgC, the PMM activity, catalyzes the conversion of mannose-6-phosphate to mannose-1-phosphate, leading to the formation of GDP-D-mannose and GDP-D-manuronic acid. These are required for A-band PS²⁰¹ and alginate synthesis^{201, 209}. It is of interest to note that *algC*, *galU* and the *rml* operon are all located outside the *waaF* core gene cluster.

Our genetic data suggest that the assembly of the two distinct outer-core glycoforms requires six glycosyltransferases, in addition to the glycosyltransferase encoded by *wapG*, which transfers the first hexose sugar to lipid A-inner core. Two genes, *wapR* and *migA*, which encode two of the six glycosyltransferases are currently under investigation in our laboratory. *wapR* encodes the α -1-3-rhamnosyltransferase that adds the rhamnose residue to the core that is required for the attachment of long chain PS whereas *migA* encodes the α -1-6-rhamnosyltransferase required for the assembly of uncapped core glycoform. These functional assignments are based on the evidence of the LPS phenotypes and complementation results observed in mutant constructs of *wapR*, *migA* in a PAO1 background and *migA* in a PAK background^{146, 200}. Interestingly, *wapR* is located within the core LPS gene cluster while *migA* maps outside this locus. The gene, *migA*, was identified as a *mucus* *inducible* gene whose promoter was specifically inducible during growth in respiratory mucus obtained from CF patients¹⁸⁸. A subsequent study revealed that *migA* is highly expressed in the CF lung environment and is

regulated by the RhlI/RhlR quorum sensing regulatory system²⁰⁰. Although two consensus *las*-box-like sequences were identified upstream of *migA*, it is not known which one or if both of the *las*-box-like sequences are required for *migA* expression. High-level expression of *migA* results in the loss of the core-plus-one O-antigen form of LPS (also referred to as semi-rough LPS), whereas a *migA* mutation results in an increased amount of semi-rough LPS²⁰⁰. Expression of the MigA protein is also increased during biofilm formation⁴⁹ and other groups have shown that there are significant changes in LPS expression in *P. aeruginosa* during a biofilm mode of growth^{19, 63}. Up-regulation of the *migA* gene in the CF lung environment appears to increase the production of the uncapped form of core OS and this may promote a cell-surface change that favors survival of the bacterium in this environment.

4. B-BAND POLYSACCHARIDE (O ANTIGEN)

4.1. Structure of O Polysaccharides

P. aeruginosa produces O-antigen polysaccharide (O-PS) that is referred to as B-band LPS¹⁵⁹. The O-PS consists of linear polymers of di- to penta-saccharide repeating units. A prominent feature of the *P. aeruginosa* O antigen is that it contains uronic acids, amino sugars and some rare sugars. For example, residues typically found in *P. aeruginosa* O antigen include: *N*-acetylated 6-deoxyhexosamines (D-quinovosamine, D- and L-fucosamine), 2,4-diamino-2,4-dideoxy-D-quinovose (D-bacillosamine) and acidic monoamino and diamino residues, D- and L-galactosaminuronic acid, 2,3-diamino-2,3-dideoxyuronic acids and 5,7-diamino-3,5,7,9-tetradeoxynonulosonic acids (pseudaminic acid)⁹⁷. The chemical structures of many *P. aeruginosa* O-PS have been studied extensively^{94, 97} and the O-PS of serotypes O5, O6 and O11 are shown in Figure 4. Structural information of the O-PS (specifically the primary structure or constituent sugar residues) and data from immunochemical investigations have led to the identification of 31 distinct O-antigen chemotypes^{98, 174}. The B-band O antigen is highly immunogenic and differences in the chemical structure of the O-PS are responsible for the serogroup specificity of the respective strains⁹⁸. Many different serological typing schemes that distinguish *P. aeruginosa* O antigens have been described and these include schemes by Habs⁷⁰, Sandvik¹⁶⁶, Verder and Evans¹⁸⁴, Meitert¹²⁶, Wokatsch¹⁹⁷, Lanyi¹⁰⁷, Fisher⁵⁸, Akatova and Smirnova¹ and Homma⁷⁷. The classification scheme currently used by most laboratories is the International Antigenic Typing Scheme (IATS), which describes 20 reference serotype strains of *P. aeruginosa*^{113, 114}. A comprehensive review of how the different classification schemes compare in type numbers and antigenic

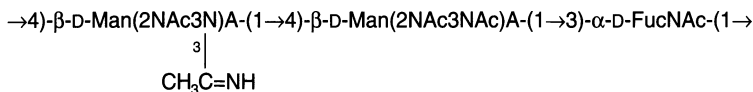
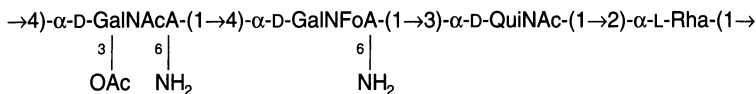
A. Serotype O5**B. Serotype O6****C. Serotype O11**

Figure 4. O-antigen structures of serotype (A) O5, (B) O6 and (C) O11. Man(2Nac3N*)A, 2-acetamido-3-acetamidino-2,3-dideoxy-mannuronic acid (* represents $\text{CH}_3\text{C}=\text{NH}$); Man(2Nac3Nac)A, 2,3-acetamido-D-mannuronic acid; FucNac, *N*-acetylfucosamine; GalNacA, *N*-acetylgalactosaminuronic acid; GalNFmA, 2-deoxy-2-formamido-galacturonic acid; QuiNac, *N*-acetylquinovosamine; Rha, rhamnose; and Glc, glucose. The *O*-acetyl and amino group substitutions are abbreviated as –OAc and –NH₂, respectively. Structures are based on refs [97], [98].

constituents of the O antigens has been provided by Stanislavsky and Lam¹⁷³. Serotyping of *P. aeruginosa* strains is normally performed using polyclonal antisera that have been adsorbed to improve specificity against a specific serotype. To provide improved specificity and sensitivity, monoclonal antibodies specific for the first 17 of the 20 IATS serotypes have been produced^{104, 105}, while polyclonal antisera against IATS serotypes 18–20 are also available¹¹⁴.

4.2. Overview of O-Antigen Biosynthesis

The biosynthesis of heteropolymeric O-antigen LPS begins in the cytoplasm with the synthesis of nucleotide-activated sugar donors. The first step in the synthesis of these nucleotide-activated sugars is the reaction between sugar-1-phosphates and their respective nucleoside triphosphates to form individual nucleoside diphosphate (NDP)-activated sugar precursors. Next, different biosynthetic pathways generate the complex sugar residues that are found in *P. aeruginosa* PSs. Once synthesized, the nucleotide-activated sugars act as donor molecules for the sequential addition of sugar residues by glycosyl-transferases onto the lipid carrier molecule, undecaprenol phosphate, to generate individual O units. These single O units are then translocated from the cytoplasmic face of the inner membrane to the periplasmic face by an O-unit translocase or flippase protein called Wzx²⁸. The O units are polymerized into the O-antigen PS by the transferase activity of the O-antigen polymerase,

Wzy⁴⁴. The chain length regulator protein called Wzz regulates the length of the PS generated by the Wzy protein³⁹. The majority of the genes encoding these proteins as well as the enzymes involved in the biosynthesis of the nucleotide-activated precursor sugars are located in one O-antigen biosynthetic gene cluster. After assembly of the PS, the O-antigen ligase, WaaL, transfers the O antigen to the core-lipid A to make the finished LPS molecule that is then transported to the outer leaflet of the outer membrane. This assembly process is based on the Wzy-dependent assembly model of O-antigen biosynthesis, which has been extensively reviewed^{150, 191}. Characterization of the O-PS assembly genes designated as *wz** in *P. aeruginosa* have been performed using mainly an approach whereby knockout mutants were constructed and the resultant LPS phenotypes were examined immunochemically and structurally to reveal that B-band LPS assembly follows the *wzy*-dependent assembly model¹⁶⁰.

4.3. Genes of the B-Band Gene Clusters

The genetics of B-band biosynthesis in *P. aeruginosa* has been reviewed¹⁶⁰. It is important to note that in many of the early studies in the literature, assignment of putative function of the gene products of the O-antigen gene cluster mainly relied on: (a) similarity to other known proteins (most of which have not been characterized at the biochemical level), (b) LPS phenotypes of null mutants, and (c) data from cross-complementation experiments with genes from other organisms. In contrast, within the past few years, our group has focused on determining the biochemical activities of the enzymes encoded by the genes in the O-antigen biosynthetic locus. The significance of this work reaches beyond the scope of *P. aeruginosa* LPS biosynthesis because many of the *P. aeruginosa* enzymes involved in sugar nucleotide biosynthesis are representative members of families of similar enzymes found in other medically important bacteria.

In general, O-antigen biosynthesis in *P. aeruginosa* is best understood in strain PAO1 (serotype O5). Strains producing O6 and O11 O antigens, however, are the most frequently encountered serotypes isolated from the hospital setting and the environment^{18, 57, 145, 180}. A report by MacIntyre *et al.*¹¹⁷ has shown that in the Polyvalent Extract Vaccine (Wellcome Biotechnology), the predominant protective component against *P. aeruginosa* infection is LPS from IATS serotype O6. The O11 serotype strains are also important in the medical community because of their association with a multidrug resistance phenotype¹⁷⁷. Thus, the O-antigen gene clusters for *P. aeruginosa* serotypes O5 and O6 have been characterized in detail by our group^{17, 26} and the O-antigen cluster of serotype O11 has been characterized by Goldberg and her colleagues⁴⁶. The gene names within the three clusters have been assigned in accordance with bacterial polysaccharide synthesis gene nomenclature¹⁵⁷.

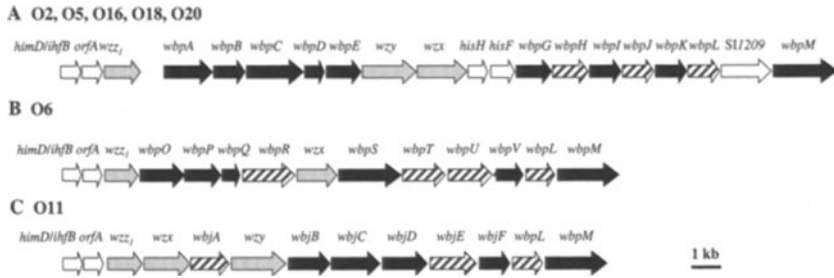


Figure 5. The genetic organization of the B-band biosynthetic gene clusters for serotypes O5, O6 and O11. Genes involved in the biosynthesis of the O unit (A) in O5 are designated *wbpA* through *wbpM*, (B) for serotype O6, *wbpO* through *wbpV* and (C) for serotype O11, *wbpA* through *wbpF*. The genes that encode the flippase, O-antigen polymerase and O-antigen chain length regulator, are designated *wzx*, *wzy* and *wzz*, respectively. Black arrows designate genes involved in the biosynthesis of nucleotide-activated precursor sugars, gray arrows represent genes involved in polymerization and assembly, hatched arrows represent genes that encode glycosyltransferases and white arrows represent ORFs whose functions have not been shown to correlate with LPS biosynthesis. All three loci are shown on the same scale; bar equals 1 kb. The assignment of gene functions are based on data from refs [17], [26], [46], [152].

Burrows *et al.*²⁶ were the first to characterize a complete cluster of genes involved in the biosynthesis of the O antigen in strain PAO1 (serotype O5). The genes that encoded the flippase, O-antigen polymerase, and O-antigen chain length regulator, were named *wzx*, *wzy* and *wzz*, respectively. Other genes involved in the biosynthesis of the O unit in O5 were designated *wbpA* through *wbpM* (Figure 5A). The O-antigen gene cluster of serotype O6, possessed ORFs that are highly homologous to *wzz*, *wzx*, *wbpL* and *wbpM* of PAO1, and were thus given the same names and presumed to have the same function. The other O-antigen genes in the O6 cluster are designated *wbpO* through *wbpV* (Figure 5B). A unique feature in the O6 cluster is the absence of an O-antigen polymerase gene, *wzy*. The serotype O11 O-antigen gene cluster contains *wzz*, *wzx*, *wxy*, *wbpL* and *wbpM* and the other genes were designated *wbpA* through *wbpF* by Dean *et al.*⁴⁶ (Figure 5C).

4.4. Genetic Diversity of the 20 B-Band O-Antigen Gene Clusters

Burrows *et al.*²⁶ showed that the 5' end of the B-band gene clusters contains the "serogroup-specific" region while the 3' end contains a conserved gene, *wbpM*. Comparison of the sequence data from serotypes O5, O6 and O11 gene clusters revealed a conserved *himD/ihfB* gene at the 5' end and

wbpM at the 3' end^{17, 26, 46}. Assuming the B-band clusters resided in a common location on the *P. aeruginosa* genome, Raymond *et al.*¹⁵² used a yeast recombinational cloning technique and successfully cloned the O-antigen gene clusters from all 20 IATS reference serotype strains. This technique has proven to be broadly applicable for targeted cloning of DNA sequences within genomic DNA, provided that the flanking sequences of the genes of interest are known and conserved. Sequencing of the 20 serotype O-PS gene clusters was successfully achieved by Raymond *et al.*¹⁵². The sequence data along with the known structures of the 20 O-PS antigens^{94, 97} have been invaluable for studies to attain an overall understanding of the relationships between the biosynthetic pathways of the different O-PS.

The boundaries of the 20 B-band gene islands are found within a sharp 20-bp sequence at the 5' end and within the *wbpM* gene at the 3' end¹⁵². The 20 B-band gene islands have mol% G+C contents ranging from 46 to 55 (see ref. [152]). This is in marked contrast to the overall mol% G+C content of the *P. aeruginosa* PAO1 genome, which is 67 (see refs. [139, 176]). The *wbpM* gene, however, has a mol% G+C content of approximately 62. This unusual discrepancy in base composition of the B-band genes is consistent with the theory that these gene clusters have been acquired by horizontal transfer from other bacterial species¹⁶⁰, whereas the *wbpM* gene may be an ancestral *P. aeruginosa* gene. In several members of the *Enterobacteriaceae*, which produce hetero-polysaccharide O antigens, the O-antigen gene cluster is also located in a conserved locus on the genome, flanked by *galF* and *gnd* at the 5' and 3' end, respectively^{11, 12, 157}. More recently, a highly conserved 39-bp element, the JUMPstart sequence (Just Upstream of Many Polysaccharide-associated gene *starts*; ref. [76]) has been used as the 5' sequence for designing primers for PCR-based cloning and sequencing of the O-antigen regions of *E. coli* O157¹⁸⁹ and several *Shigella* serotypes³³. So far, this JUMPstart sequence has not been localized near the 5' end of the *P. aeruginosa* B-band gene clusters.

Cloning and sequencing of the O-antigen biosynthetic loci from the 20 IATS reference strains by Raymond *et al.*¹⁵² revealed 11 distinct gene clusters that are highly divergent from one another at the DNA sequence level. Each of the B-band gene islands from serotypes O1, O4, O6, O9 and O12 is a distinct gene cluster. Other distinct gene clusters are the ones shared by two O serotypes including O3 and O15, O7 and O8, O10 and O19, O11 and O17, O13 and O14. B-band gene islands of serotypes O2, O5, O16, O18 and O20 are 98% identical; thus, they belong to a single group¹⁵². This observation substantiates previous results based on Southern hybridization²⁶ and serotyping experiments using O-specific and cross-reactive monoclonal antibodies¹⁰². Enzymes expressed by genes that map outside the B-band gene cluster likely contribute to the chemical variability of the O-antigen structures within these groups. However, there are a few strains that produce O antigen, as shown by positive reactivity to serotyping

antibodies, but contain what appears to be a non-functional B-band gene cluster in the *himD/ihfB-wbpM* region¹⁵². For example, IATS reference strain 33362 produces O15 O antigen, yet the *himD/ihfB-wbpM* region is devoid of a complete O-antigen gene cluster. Instead, there is only a small gene, *orfA* (which is most similar to the *orfA* of serotype O11) and the 3' end half of the *wbpM* gene. A second IATS O15 reference strain obtained from the strain collection of Dr. Stephen Lory (Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA) also produced O15 O antigen, yet within the *himD/ihfB-wbpM* region there was a copy of the serotype O3 gene cluster that was interrupted in three different genes by insertional elements. This cluster is thought to be non-functional. A non-functional B-band gene cluster has also been identified in IATS reference strain O17 by two independent groups^{48, 152}. The cryptic gene cluster in the *himD/ihfB-wbpM* region is that of serotype O11 and is punctuated with insertion elements. It is interesting that both of the residual B-band gene clusters in the serotype O15 and O17 strains are of O11 origin. These data imply that the genes involved in B-band synthesis for these strains reside elsewhere on the chromosome. Interestingly, the genome of *P. aeruginosa* strain PAO1 contains not only the *wbp* O5 B-band gene cluster, which is located on the genome at ORFs PA3160-PA3141, but also contains two other putative gene clusters involved in polysaccharide synthesis located at ORFs PA1381-PA1393 and PA2231-PA2240¹²² (PseudoCAP [<http://www.nature.com/nature/journal/v406/n6799/extref/406959ai6.jpeg>]; Pseudomonas Genome Project¹⁷⁶).

The B-band gene clusters of serotypes O2, O5, O16, O18 and O20 are 98% identical¹⁵², thus, the subtle variations in the structures among these serotypes must be due to genes that are unlinked to this locus. Modifications of the O-PS have been shown to occur by the action of lysogenic bacteriophages. For example, bacteriophage D3, which lysogenizes *P. aeruginosa* strain PAO1, causes two major changes in the O5 O antigen. Firstly, the C-4 position of the FucNAc residue is *O*-acetylated (characteristic of serotype O20), and the linkage between the O units is changed from α 1-4 to β 1-4 (change from serotype O5 to O16)¹⁰¹. The bacteriophage D3 genome contains three genes required for "serotype conversion," an inhibitor of the α -polymerase (*iap*); an *O*-acetylase (*oac*); and a β -polymerase (*wzy β*)¹³². Transformation of the *iap* gene into PAO1 and other serotype O5 strains showed inhibition of Wzy activity in vivo and allows the phage encoded β -polymerase to form new β -linked B-band LPS. The phage-encoded *Iap* inhibitor is a novel peptide because it is capable of inhibiting B-band LPS containing α -linked O units in different *P. aeruginosa* strains. Investigations are underway in our laboratory to understand the mechanism of action of this PS inhibitor because it may provide valuable information for the development of potential LPS inhibiting drugs.

4.5. Conserved Proteins of the B-Band Gene Clusters

Analysis of the 20 IATS B-band gene clusters by Raymond *et al.*¹⁵² revealed that although there is a lack of conservation at the nucleotide sequence level, there is a certain degree of conservation in the organization of the genes. This is an intriguing observation considering the differences in chemical structures of the sugar residues that exist between the O serotypes. Located at the 5' end of each gene cluster is a *wzz* gene, which encodes the O-antigen chain length regulator protein. Two distinct Wzz proteins, Wzz1 and Wzz2, have been identified in *P. aeruginosa* strain PAO1 (serotype O5)^{27, 39}. *wzz1* is located at the 5' end of the B-band gene cluster, while *wzz2* maps outside this locus. Wzz1 regulates the production of two preferred O-antigen chain lengths in serotype O5 that equate to 12–16 and 22–30 O-antigen repeat units, whereas Wzz2 imparts the chain length of approximately 40–50 repeats. Daniels *et al.*³⁹ investigated the relative distribution of O5-specific Wzz1 and Wzz2 within the 20 serotypes and found that serotype strains O1, O2, O5, O16, O18 and O20 express a Wzz1 protein that is unique to this cross-reactive group, while Wzz2 is conserved among all 20 IATS reference strains. Based on the sequence data of *P. aeruginosa* B-band O-antigen gene clusters by Raymond *et al.*¹⁵², we now know that the serotype-specific *wzz1* gene is located at the 5' end of each of the 20 O-antigen clusters.

O-PSs from the 20 different serotypes exhibit different chain length distributions¹⁰³ and the exact mechanism by which Wzz proteins function in controlling modal chain length is not completely understood. Two different models have been proposed to describe the Wzz-dependent chain length regulation. The first model proposed by Bastin *et al.*¹¹ indicates that Wzz acts as a “timing clock” to modulate chain length during its interaction with Wzy polymerase, whereas the second model described by Morona *et al.*¹³¹ suggests that Wzz acts as a molecular chaperone in a complex with Wxy and WaaL to control O-PS chain length. Furthermore, it has been postulated that modal chain length is determined at the ligation step¹⁹². This would require the interaction of Wzz and Wzy with the O-antigen ligase (WaaL) so that PS of the preferred chain length is ligated to the lipid-A core. In contrast to this postulate, Daniels *et al.*³⁹ showed that modal chain length-distributed O-PS antigen in *P. aeruginosa* strain PAO1 (serotype O5) occurs on the growing undecaprenylpyrophosphate-bound O-antigen polymer, before ligation to the core-lipid A. In addition, by performing immunoprecipitation and Western immunoblotting experiments, our group has provided the evidence to show that both Wzz1 and Wzz2 proteins are associated with the growing O-antigen polymer, which is covalently attached to the undecaprenol phosphate³⁹. To define the molecular mechanisms of regulation of O-antigen chain lengths, other approaches will be attempted in future experiments including overexpression of the proteins, protein

purification and the use of biophysical methods to measure protein–protein interactions.

The 3' end of the B-band gene clusters contain four genes of conserved gene order among most of the 20 serotype LPS gene islands. These four genes encode proteins designated as WbpH, WbpK, WbpL and WbpM in serotype O5²⁶, WbpU, WbpV, WbpL and WbpM in serotype O6¹⁷ and WbjE, WbjF, WbpL and WbpM in serotype O11⁴⁶. Raymond *et al.*¹⁵² annotated these proteins according to the *Protein families* database of alignments and HMMs (Pfam; refs [13], [14]) and their results showed that WbpH, WbpU, WbjE and WbpL belong to glycosyltransferase families while WbpK, WbpV, WbjF and WbpM belong to the NAD-dependent epimerase/dehydratase family and the polysaccharide biosynthesis protein family, respectively (a complete list of the annotated proteins can be found at the website for the University of Washington Genome Center at <http://www.genome.washington.edu/uwge/O-antigen/>). The putative functions of these proteins in the synthesis of each of the three different O antigens has been described previously^{17, 26, 46}. Functional assignment to genes based on *in silico* analysis can only be regarded as “putative” unless more experimental data can be obtained to substantiate such assignments. Therefore, our group has been focusing on performing in-depth genetic and biochemical characterization to determine their functions. It is important to note that even a single family of proteins may contain enzymes with a wide variety of functions. Since mutations in *wbpH*, *wbpK*, *wbpL* and *wbpM* have been shown to be essential for O-antigen synthesis in strain PAO1 (serotype O5)^{26, 160, 161}, the individual functions of the gene products and their homologues in the other *P. aeruginosa* serotype strains have been further characterized.

WbpL is a unique enzyme because it is a bifunctional glycosyltransferase that initiates assembly of both B-band O antigen and A-band common antigen. *P. aeruginosa wbpL* mutants constructed in serotypes O2, O5, O6, O7 and O16 backgrounds showed the requirement of this gene in the production of both B-band and A-band PS^{17, 50, 161}. To begin assembly of the individual O units, WbpL transfers the first sugar residue of the O unit to the undecaprenol phosphate lipid carrier (Und-P), and this sugar-PP-Und acts as a scaffold for the sequential addition of sugars by the O-antigen glycosyltransferases. To initiate assembly of A-band common antigen, WbpL transfers GlcNAc-1-P to Und-P¹⁶¹ and GlcNAc-PP-Und is the acceptor for the sequential addition of D-rhamnose by A-band specific glycosyltransferases to assemble the common antigen. Evidence to support this hypothesis includes the similarity of WbpL to WecA from *E. coli*. WecA is a GlcNAc-1-phosphate transferase that transfers GlcNAc-1-P from UDP-GlcNAc to Und-P to initiate O-PS synthesis as well as enterobacterial common antigen in *E. coli*^{2, 4, 5, 125}. Cross-complementation experiments show that *wecA* can complement A-band but not B-band synthesis in a *P. aeruginosa wbpL* mutant¹⁶¹. WbpL from the different serotypes must

have different sugar specificities since the first sugar residues of the O units of the different serotypes vary. Substrate specificity of WbpL from serotype O5 (WbpL_{O5}) and O6 (WbpL_{O6}) was investigated using cross-complementation experiments. The first sugar of the O unit of serotype O5 and O6 are D-FucNAc and D-QuiNAc, respectively. *wbpL*_{O5} could completely restore A-band and B-band synthesis in the serotype O6 *wbpL* mutant strain. In contrast, although *wbpL*_{O6} could completely complement A-band synthesis in the serotype O5 *wbpL* mutant, it could only partially complement B-band synthesis¹⁷. The WbpL_{O6} enzyme appears to be less efficient at recognizing UDP-D-FucNAc as a donor, while WbpL_{O5} readily recognizes both UDP-D-FucNAc and UDP-D-QuiNAc. The relaxed specificity of WbpL appears to be similar to that of *E. coli* WecA, which can initiate polymers by the formation of Und-PP-GalNAc as well as Und-PP-GlcNAc in some *E. coli* strains⁶.

The gene that encodes the glycosyltransferase that acts after WbpL in the assembly of the O unit corresponds to *wbpH*, *wbpU* and *wbjE* from serotypes O5, O6 and O11, respectively. In many O-antigen gene clusters from other organisms, the genes that encode glycosyltransferases are organized in the opposite order to which the enzyme would act^{155, 161, 168}. For example, in a trisaccharide O unit assembled on Und-P, the gene closest to the 3' end of the gene cluster that encodes a glycosyltransferase would transfer the first sugar to Und-P, and in the case of *P. aeruginosa*, has been shown to be WbpL. The glycosyltransferase that acts after WbpL to transfer the second sugar residue is proposed to be encoded by a gene slightly upstream of *wbpL* in the operon, such as *wbpH* in serotype O5. Although a null mutation in *wbpH* of serotype O5 abrogates the production of O antigen, there is no further data to substantiate which sugar residue it transfers. Knirel⁹⁴ has reported the structures of the O-PS of many different serotypes of *P. aeruginosa*; however, the exact order of the sugars within an O-PS unit had not been resolved in that report, making it difficult to predict the enzyme-substrate reactions that would occur. Recently, the structure and order of the sugars within the O unit and attachment site of the O unit to the core OS for serotypes O5, O6 and O11 have been determined^{30, 45, 164} and this information is crucial for the design of glycosyltransferase assays to prove transferase functions. It is interesting that despite the differences in sugar residues between the serotypes, WbpL and the second glycosyltransferase to assemble the O unit upon Und-P are relatively conserved.

wbpM defines the 3' boundary of the B-band gene islands. Its gene product, WbpM, has been overexpressed, purified and characterized at the biochemical level. This protein is a bifunctional UDP-GlcNAc C-6 dehydratase/C-4 reductase that converts UDP-D-GlcNAc to UDP-D-QuiNAc^{36, 38}. It is thought that WbpK then catalyzes the conversion of UDP-D-QuiNAc to UDP-D-FucNAc and it is D-FucNAc that is required for serotype O5 O antigen^{17, 26}. Biochemical data support the requirement of *wbpM* for the

synthesis of UDP-D-QuiNAc, UDP-D-FucNAc and its derivatives, including 4-amino-D-QuiNAc {4-amino-2,4,6-trideoxy-*N*-acetylglucosamine [UDP-D-Bac (2NAc4N)]}. Interestingly, all 20 serotypes of *P. aeruginosa* contain *wbpM* although the O antigens are structurally distinct²⁹. A mutation in *wbpM* in serotypes O3 and O10, which contain D-Bac(2NAc4N) in the O antigen, abrogates B-band synthesis. In contrast, in serotypes O15 and O17, which do not contain D-QuiNAc, D-FucNAc or D-Bac(2NAc4N) in their LPS, *wbpM* mutation in these serotypes has no effect on B-band LPS production. These data suggested that while all 20 serotypes possess *wbpM*, it is important for the serotypes possessing either QuiNAc or FucNAc as constituents of the O antigen but it is not universally required for O-antigen biosynthesis. Homologous genes of *wbpM* have been identified in *Bordetella pertussis* (*wblL*), *Staphylococcus aureus* (*cap8D/cap5D*), *Yersinia enterocolitica* (*wbcP*) and *Helicobacter pylori* (*flaA1*). Each of these WbpM homologues could complement a *P. aeruginosa* PAO1 *wbpM* mutant to restore B-band O-antigen production^{29, 37}. Biochemical studies of *H. pylori* FlaA1³⁷ and *P. aeruginosa* WbpM³⁸ revealed that both proteins have identical functions catalyzing C-4 and C-6 dehydration of the substrate, UDP-*N*-acetylglucosamine (UDP-GlcNAc), to yield UDP-QuiNAc as a reaction product.

Two genes required for O antigen Wzy-dependent assembly are *wzy* and *wzx*, which encode the O-antigen polymerase and O-unit translocase, respectively. At the nucleotide levels, *wz** genes (the asterisk represents either *x*, *y*, or *z*) do not share sequence homology with other genes; therefore, they are usually localized in the “serotype-specific” region of the LPS gene clusters. However, the proteins encoded by these genes, namely, Wzy and Wzx, in other organisms have been shown to be highly hydrophobic with 11–13 and 12 potential membrane-spanning domains, respectively^{40, 112, 130, 156}. This provided the clue to identify putative *wz** genes in *P. aeruginosa* LPS clusters. By performing hydropathy scans of the *P. aeruginosa* LPS gene clusters, ORFs that could potentially encode Wzy and Wzx proteins in all 20 clusters have been identified, with only serotype O6 as the notable exception^{17, 152}, that is, *wzy* presumably maps outside the O-antigen locus in the genome of serotype O6 strains.

A *wzy* gene was cloned from *P. aeruginosa* strain PAO1 (serotype O5). Its function as an O-polymerase gene became apparent when a knockout *wzy* mutant exhibited the characteristic “semi-rough LPS” phenotype. LPS from the *wzy* mutant is composed of lipid A-core capped with a single O-antigen unit^{41, 44} and similar results were observed in a subsequent study by Dean *et al.* on serotype O11^{45, 46}. Due to the presence of multiple membrane-spanning domains in this gene, protein expression of Wzy was expected to be difficult. Thus far, there has not been any report on the purification of this protein.

To characterize the function of *wzx*, a null mutant of this gene was constructed in *P. aeruginosa* PAO1 background; and consistent with our

hypothesis, LPS prepared from the *wzx* mutant was totally devoid of B-band LPS. Interestingly, the *wzx* mutation also led to a marked delay in the production of A-band LPS. This was not expected, since A-band LPS is composed of a homopolymer of D-rhamnose whose biosynthesis follows the ABC-transporter-dependent model¹⁶⁰. Normal production of A-band LPS was restored when the *wzx* mutant was supplied with *wbpL* *in trans*. Burrows and Lam²⁸ proposed that the delay in A-band production might be due to insufficient access to the glycosyltransferase WbpL, which may arise when the completed B-band O unit is not successfully translocated to the periplasm. Since WbpL is also required for the initiation of A-band production, without adequate amounts of WbpL, assembly of A-band LPS is delayed. Evidence from studies on the WbpL homologues, *S. enterica* WbaP and *E. coli* WecA support the observation that these enzymes might play a role in the release of Und-PP-linked intermediates prior to being flipped to the periplasm, in addition to their role in initiation of PS synthesis^{5, 112, 150}.

5. BIOSYNTHETIC PATHWAYS OF HEXOSE RESIDUES OF THE O ANTIGEN

UDP-*N*-acetyl-D-glucosamine (UDP-D-GlcNAc) is a common precursor for the biosynthesis of *N*-acetyl dideoxy- and deoxysugars. To perform biochemical characterization of the enzymes involved in the biosynthesis of *P. aeruginosa* B-band LPS, we have used a number of methods either individually or in combination. A *p*-dimethylaminobenzaldehyde (DMBA) colorimetric assay for the detection of *N*-acetylated nucleotide-activated sugars was developed for analyzing the activity of WbpP. In addition, we also utilized a number of cutting-edge techniques including capillary electrophoresis (CE), mass spectrophotometry (MS) and nuclear magnetic resonance (NMR). The method of CE provides exquisite resolution for the separation of closely related compounds including epimers and isomers of sugar nucleotides. Using the aforementioned techniques, we were able to characterize the enzymes involved in the pathways for the biosynthesis of *N*-acetyl dideoxy/deoxy hexoses including UDP-*N*-acetyl-D-galactosamine (UDP-D-GalNAc)³⁵, UDP-*N*-acetyl-D-galactosaminuronic acid (UDP-D-GalNAcA)²⁰⁵, UDP *N*-acetyl-D-quinovosamine (UDP-D-QuiNAc)³⁶, UDP-*N*-acetyl-L-fucosamine (UDP-L-FucNAc)⁹³ and UDP-*N*-acetyl-L-quinovosamine (UDP-L-QuiNAc)⁹¹ (Figure 6).

It is intriguing to observe that a majority of the proteins involved in these pathways belong to a large family of proteins called short-chain dehydrogenases/reductases (SDR). The enzymes belonging to this family include dehydratases, dehydrogenases, epimerases, isomerases and reductases^{84–86, 142}. Typically, these proteins contain 250–350 amino acid residues and catalyze the

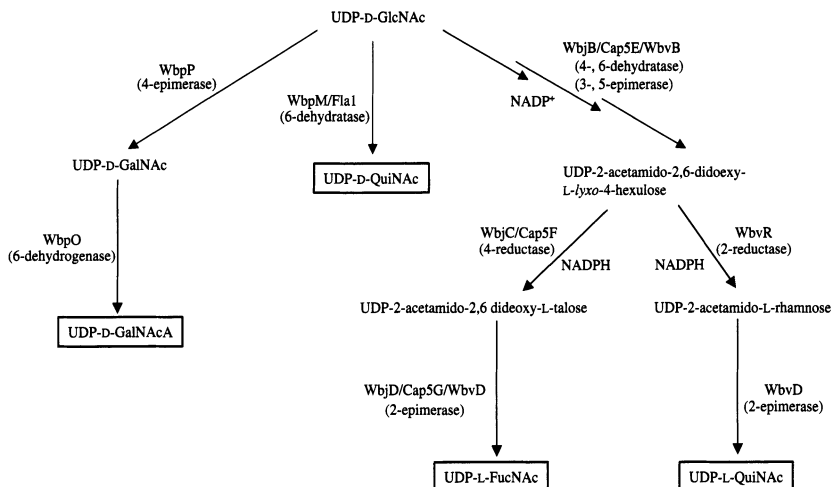


Figure 6. Biosynthetic pathways that have been characterized to date for nucleotide-activated sugars, L-QuiNAc (*N*-acetyl-L-quinovosamine), L-FucNAc (*N*-acetyl-L-fucosamine), L-RhaNAc (*N*-acetyl-L-rhamnosamine), D-GalNAc (*N*-acetyl-L-galactosamine), D-GalNAcA (*N*-acetyl-L-galactosaminuronic acid), from precursor UDP-D-GlcNAc (*N*-acetyl-D-glucosamine). The pathways were drawn based on data from refs [35], [36], [91], [93], [205].

oxidation/reduction reactions with a cofactor NAD(P)(H) binding domain at the amino terminus¹⁴². There is low homology at the primary amino acid level among the enzymes in the SDR family. However, SDR family members invariably possess a nucleotide-binding signature motif, GXXGXXG (G is Gly, and X can be any amino acid) referred to as a Rossmann fold. As a result, SDR enzymes share similar tertiary structures at the cofactor-binding site. Another signature motif that they share is YXXXXK (Y is Tyr and K is Lys) which is found at the active site of these enzymes¹⁴². The carboxyl terminus of these proteins is generally varied due to the particular substrate that they catalyze.

5.1. Synthesis of UDP-*N*-Acetyl-D-Galactosaminuronic Acid

Two of the four residues of the serotype O6 O antigen are galactosaminuronic acid (α -D-GalNAcA) derivatives and α -D-GalNAcA is one of the most common acidic sugars identified in the LPS or capsular polysaccharides of numerous bacterial pathogens. Some examples of α -D-GalNAcA containing polysaccharides include: The O-PS of *P. fluorescens* biovar B¹⁶⁹, *Shigella*-like *E. coli* O121¹⁴⁰, *Acinetobacter haemolyticus*⁷³ and *Vibrio anguillarum*¹³⁸, as well as capsular polysaccharides of *S. enterica* serovar Typhi⁷⁴, *S. aureus*¹¹¹, and *Vibrio vulnificus*^{153, 154}.

In *P. aeruginosa* serotype O6, the *N*-acetyl-D-galactosaminuronic acid (GalNAcA) residue of the O antigen was postulated to be derived from a nucleotide-activated form, namely, UDP-D-GalNAcA. Accordingly, this precursor sugar should be synthesized in two steps, starting with UDP-D-GlcNAc as a substrate, which will be epimerized to form UDP-D-GalNAc. This is followed by an oxidation reaction at C-6 to convert UDP-D-GalNAc into UDP-D-GalNAcA. Two candidate genes that could encode the enzymes for the two-step pathway were identified as *wbpP* and *wbpO* in the O6 LPS gene cluster¹⁷. The products of these genes, WbpO and WbpP, show significant homology to *S. enterica* serovar Typhi WcdA and WcdB, respectively. WcdA and WcdB are required for the synthesis of the Vi polysaccharide, which is a homopolymer of α -1,4, 2-deoxy-2-*N*-acetylgalactosaminuronic acid (GalNAcA)^{74, 185, 190}. B-band LPS production is abrogated when a mutation occurs in either *wbpP* or *wbpO* in *P. aeruginosa* strain PAK (serotype O6). This LPS deficiency is fully restored after complementation with the *wcdB* or *wcdA* gene, respectively¹⁷. In addition, WbpP shows 23% identity with the C-4 UDP-D-glucose epimerase, GalE, from *E. coli* and both of these proteins as well as other putative epimerases belong to the enzyme SDR family.

To conduct biochemical characterization of WbpP, the protein was overexpressed and purified. Using CE, the activity of WbpP was shown to utilize UDP-D-GlcNAc to generate a new peak. This product peak migrated to the same area as the UDP-D-GalNAc standard sample, and proved that WbpP catalyzed the C-4 epimerization of the substrate to produce UDP-D-GalNAc. To facilitate kinetic measurements of the C-4 epimerase activity involving UDP-D-GlcNAc and UDP-D-GalNAc as substrates, a spectrophotometric assay using DMAB was developed. This assay allowed quantitative assessment of the kinetics of WbpP activities and its substrate specificity. Although WbpP showed similar K_m values for binding UDP-D-Glc and UDP-D-Gal as compared to binding UDP-*N*-acetylated sugars, the catalysis of non-acetylated substrates is *c.* 1,000-fold less efficient than with acetylated ones (Table 1). Therefore, WbpP is specific for *N*-acetylated substrates UDP-D-GlcNAc and UDP-D-GalNAc³⁵. An epimerization reaction is bi-directional, and at equilibrium, both the substrate and the product will be detectable in the reaction mixture. It was of interest to note that in vitro and at equilibrium of the enzyme-substrate reaction, 70% of UDP-D-GalNAc is converted to UDP-D-GlcNAc. In contrast, when UDP-D-GlcNAc was used as the substrate, 30% conversion to the product UDP-D-GalNAc was achieved. These findings correlate well with the K_m values obtained, whereby binding of UDP-D-GlcNAc to the enzyme was less efficient than that of UDP-D-GalNAc (Table 1). Therefore, in vitro, the equilibrium is shifted toward the production of UDP-D-GlcNAc, and a shift of the equilibrium toward the production of the glucose isomer has previously been reported for *E. coli* GalE¹⁹⁶. Thus, we proposed that in vivo, the next enzyme involved in the B-band LPS

Table 1. Kinetic parameters for WbpP, WbpO_{Rf}, FlaA1, WbpM and their mutants as established by CE.

Proteins	K_m (mM)	k_{cat} (min ⁻¹)	k_{cat}/K_m (mM ⁻¹ min ⁻¹)	Conditions	References
WbpP ^a	224 ± 17	120 ± 3	536 ± 57	pH 7, 37 °C	[35]
WbpP ^b	197 ± 15	271 ± 7	1375 ± 143	pH 7, 37 °C	[35]
WbpO _{Rf} ^{a,c}	22.2 ± 1.2	5.5	0.25	pH 8.5, 37 °C	[205]
WbpO _{Rf} ^b	7.79 ± 0.19	47.8	6.13	pH 8.5, 37 °C	[205]
FlaA1 (wild type) ^a	159 ± 15	5.7 ± 0.5	335.9 ± 6.7	pH 7, 37 °C	[37]
WbpM (His-S262) ^a	2770 ± 6	168 ± 14	58.0 ± 5.0	pH 10, 30 °C	[36]
WbpM 438Y ^a	1423 ± 48	55.0 ± 5	39.0 ± 2.0	pH 10, 30 °C	[38]
FlaA1 Y141M ^a	565 ± 6	1.7 ± 0.0	3.1 ± 0.1	pH 7, 37 °C	[38]
FlaA1 Y141M ^a	251 ± 5	3.9 ± 0.1	15.3 ± 0.5	pH 10, 37 °C	[38]

^aUDP-D-GlcNAc was used as the substrate.^bUDP-D-GalNAc was used as the substrate.^cThe “Rf” designation in WbpO_{Rf} refers to the refolded enzyme.

biosynthetic pathway is responsible for pulling the reaction equilibrium toward the production of UDP-D-GalNAc³⁵.

Two pieces of evidence indicated that WbpO is the enzyme that catalyzes the next reaction step in the pathway to convert the product of the WbpP reaction, UDP-D-GalNAc, to UDP-D-GalNAcA. First, *wbpP* and *wbpO* genes are contiguous in the O6 B-band LPS gene locus, and second, sequence analysis showed that WbpO has homology to other dehydrogenases. For biochemical characterization, WbpO was overexpressed and purified to homogeneity. No enzymatic activity could be detected with the soluble form of this protein suggesting that WbpO might be folded incorrectly during expression. Therefore, WbpO was purified under denaturing conditions and refolding techniques were optimized to obtain enzymatically active WbpO²⁰⁵. Differences in the secondary structure between the soluble WbpO (nonactive) and refolded WbpO (active) proteins were detected by circular dichroism analysis and these data implied that the soluble and inactive WbpO is folded incorrectly. The refolded WbpO enzyme was used for detailed biochemical characterization. Since the predicted product of the reaction, UDP-D-GalNAcA, is not commercially available, there was no standard to confirm the identity of the reaction products. Therefore, it was necessary to couple CE with mass spectrometry and tandem mass spectrometry (CE-MS/MS) to identify the reaction products. The results obtained by this approach equivocally demonstrated that WbpO catalyzed the conversion of UDP-D-GalNAc to UDP-D-GalNAcA. Kinetic investigation of WbpO showed a preference of WbpO to UDP-D-GalNAc over UDP-D-GlcNAc as the substrate. Moreover, the binding efficiency of UDP-D-GalNAc to the enzyme is about 25 times higher than that

for UDP-D-GlcNAc. The activity of WbpO for UDP-D-Gal, the nonacetylated form of UDP-D-GalNAc, was insignificantly low. These data revealed that WbpO is specific for the *N*-acetylated substrate, with a clear preference for UDP-D-GalNAc over UDP-D-GlcNAc. WbpO was the first UDP-GalNAc dehydrogenase to be characterized at the molecular and biochemical level²⁰⁵. By achieving the enzymatic characterization of WbpO, we have solved the two-step biosynthetic pathway of UDP-D-GalNAcA and provided the evidence to prove the hypothesis that we had put forth earlier, that in vivo in *P. aeruginosa*, a second enzymatic step is pulling the equilibrium toward UDP-D-GalNAc formation in the first step, despite a preference for the reverse direction to form UDP-D-GlcNAc in the C-4 epimerization reaction catalyzed by WbpP.

5.2. Biosynthesis of UDP-*N*-Acetyl-L-Fucosamine

N-acetyl-L-fucosamine (L-FucNAc) is a constituent of the O antigen of *P. aeruginosa* serotypes O4, O11 and O12⁹⁷, the O antigen of *E. coli* serotype O26¹¹⁸, the capsule polysaccharide of *Bacteroides fragilis*⁸⁸ and of Gram-positive bacteria *Streptococcus pneumoniae* type 4⁸³ and *Streptococcus aureus* serotype 5 and 8 (see refs [60], [129]). It is important to note that the clinical isolates of *P. aeruginosa*, *S. aureus* and *S. pneumoniae* produce PS that contain L-FucNAc^{3, 145, 170}. The three genes within the *P. aeruginosa* O11 O-antigen gene cluster proposed to be involved in the biosynthesis of UDP-L-FucNAc are WbjB, WbjC, WbjD, which show 67%, 40% and 57% identity to *S. aureus* Cap5E, Cap5F, Cap5G and 62%, 42% and 51% identity to Orf13, Orf14 and Orf15 of *S. pneumoniae*, respectively^{46, 82, 167}.

In two separate studies, UDP-*N*-acetylmannosamine (UDP-D-ManNAc) was proposed to be the precursor for the biosynthesis of UDP-L-FucNAc^{82, 108}. Jiang *et al.*⁸² proposed a three-step pathway involving (a) a C-2 epimerization step in which UDP-D-GlcNAc is converted to UDP-D-ManNAc, followed by (b) a dehydration step to form UDP-4-keto-6-deoxy-D-ManNAc and (c) an epimerization and oxidoreduction step to form UDP-L-FucNAc. Lee and Lee¹⁰⁸ also suggested a three-step pathway with UDP-D-ManNAc as the starting precursor. They postulated that UDP-D-ManNAc is first synthesized by the gene product of *mnaA* (not present in *P. aeruginosa* O11 or *E. coli* O26) and involves the following steps catalyzed by three enzymes, (a) C-3 epimerization, (b) C-5 epimerization and (c) C-6 dehydration to form UDP-L-FucNAc. Recently, our laboratory has provided genetic and biochemical evidence to show that the three proteins WbjB/WbjC/WbjD of *P. aeruginosa* serotype O11 are functional homologues of Cap5E/Cap5F/Cap5G of *S. aureus* and more importantly, these proteins possess enzymatic activities that use a different pathway than the aforementioned ones to synthesize UDP-L-FucNAc⁹³.

WbjB, WbjC and WbjD were overexpressed and purified to homogeneity. The first enzyme in the UDP-L-FucNAc pathway is WbjB. Based on the two pathways previously proposed by Jiang *et al.*⁸² and Lee and Lee¹⁰⁸, UDP-D-ManNAc is a common precursor for UDP-L-FucNAc biosynthesis. No reaction could be observed when UDP-D-ManNAc and WbjB were added to the enzyme–substrate mixture. This observation is consistent with the fact that PA103 does not have the gene *mnaA* to produce UDP-D-ManNAc in vivo. Since several enzymes that we have examined utilize UDP-D-GlcNAc as a substrate, we were curious to test whether WbjB might also use the same substrate for its enzymatic activity. Putting WbjB and UDP-D-GlcNAc in an enzyme–substrate reaction revealed that WbjB is capable of completely converting UDP-D-GlcNAc into two detectable peaks as visualized by CE. WbjB requires cofactor NADP⁺, but not NAD⁺. In addition, WbjB is specific for converting UDP-D-GlcNAc and cannot utilize UDP-D-GalNAc and UDP-D-Glc as substrates. Time course studies show that the two peaks were in a ratio of 1 : 3 regardless of the incubation time, which is typical of epimerase reactions. The keto-intermediate products of WbjB were further analyzed by CE-MS which indicated that one product had a mass that is consistent with the C-4, C-6 dehydration product. CE-MS/MS analysis of the second reaction peak indicated that this product is UDP-2-acetamido-4-keto-3,6-dideoxy sugar. The first keto-intermediate which migrated faster than NADP⁺ was purified by HPLC using an anion exchange column, lyophilized and analyzed by high-field NMR analysis to elucidate the chemical structure of the product. The product was determined to be a sugar nucleotide UDP-2-acetamido-2,6-dideoxy- α -D-xylo-4-hexulose. The second intermediate was somewhat unstable; however, we were able to lyophilize this product following removal of the enzyme in the mixture by ultrafiltration and upon analysis by NMR, this second intermediate was shown to be UDP-2-acetamido-2,6-dideoxy- β -L-lyxo-4-hexulose.

To test the enzymatic activities of the other Wbj proteins, UDP-D-GlcNAc was incubated in a “coupled” two-step reaction with WbjB and WbjC in the presence of cofactor NADP⁺ and NADPH. The emergence of a new peak that comigrated with UDP-D-GlcNAc was observed. This product peak coincides with a decrease in the two peaks observed in the WbjB reaction alone on the capillary electropherogram. This reaction product was obtained by either using NADH as a cofactor, or by performing sequential reactions using WbjB, followed by WbjC with NADPH as a cofactor. To clearly identify the structures of the products from the enzyme–substrate reactions, the product peaks identified in CE-MS experiments were subjected to structural analysis with NMR. The new peak from the coupled WbjB/WbjC reaction was shown to be UDP-2-acetamido-2,6-dideoxy- β -L-talose (UDP-L-TalNAc). The structural identity of this product also revealed that WbjC, the second enzyme in the pathway, has the activity of a C-4, C-6 dehydratase.

Based on sequence analysis, WbjD showed homology to C-2 epimerases. This is a logical fit with the final step of the cascade reaction, which requires an epimerization at C-2 of the product of the previous step, UDP-L-TalNAc, to produce UDP-*N*-acetyl-L-fucosamine (UDP-L-FucNAc). Incubation of UDP-L-TalNAc with WbjD resulted in a new peak separable by CE that demonstrated identical mass and fragmentation patterns by CE-MS/MS to UDP-L-TalNAc. These results are consistent with WbjD-mediated 2-epimerization of UDP-L-TalNAc to UDP-L-FucNAc. In both cases, the *S. aureus* homologues, Cap5E, Cap5F and Cap5G were purified and activities were also examined⁹³. The activities of these proteins were identical to those of WbjB/WbjC/WbjD in *P. aeruginosa*, with a minor exception in that Cap5G showed optimal activity at 30°C vs 37°C for WbjD.

In silico analysis of proteins at the primary amino acid level suggest that homologous proteins may have the same function. The assignment of an enzyme function based on this approach is "putative," at best, and often subject to error until the biochemical characterization of the enzyme can be achieved. Results from several studies conducted in our laboratory have clearly demonstrated that proteins that are similar at the primary amino acid level have very different enzyme activities.

This is particularly evident in the UDP-L-FucNAc pathway involving WbjB–WbjC–WbjD or its homologues in *S. aureus* Cap5E–Cap5F–Cap5G, in which the initial substrate is UDP-D-GlcNAc as demonstrated by Kneidinger *et al.*⁹³ and not UDP-D-ManNAc as suggested by other researchers^{82, 108}. This kind of comparisons between assigning putative function based on sequence data and assigning function based on biochemical data was substantiated by the results of another study in which WbvB, WbvR and WbvD in *V. cholerae* O37 were found to be homologous to WbjB, WbjC and WbjD respectively⁹¹. Therefore, it would be simple to predict that the *V. cholerae* proteins would share similar function as the homologues found in *P. aeruginosa* O11 for the biosynthesis of UDP-L-FucNAc. However, two pieces of evidence suggested that the pathway catalyzed by WbvB, WbvR and WbvD might be different than that for WbjB, WbjC and WbjD. First, WbvR is only weakly homologous to WbvC, and second, the O-antigen sugar structure of *V. cholerae* O37 contains L-QuiNAc rather than L-FucNAc. To compare the products of the enzyme–substrate reactions catalyzed by these two groups of proteins, WbvB, WbvR and WbvD were overexpressed and purified to homogeneity⁹¹. WbvB was capable of utilizing UDP-D-GlcNAc as substrate and produced similar product peaks in CE-MS/MS analysis as those observed for WbjB. Interestingly, WbvR possesses reductase activity and converts the product of WbvB into a new product as opposed to UDP-L-TalNAc produced by WbjB–WbjC coupled reactions. Using NMR, the structure of this new product of WbvB–WbvR coupled reactions was solved and was shown to be UDP-*N*-acetyl-L-rhamnosamine

(UDP-L-RhaNAc). Adding WbvD of *V. cholerae* O37 to the consecutive reaction mixtures of WbvB and WbvR resulted in the formation of a C-2 epimer of UDP-L-RhaNAc and the product is UDP-2-acetamido-2,6-dideoxy-L-glucose (UDP-L-QuiNAc).

What we have learned from these comparative pathway studies in determining the function of proteins from two distinct organisms include the following: first, to predict functions of genes associated with polysaccharide biosynthesis, we require the knowledge of the organization of the cluster of genes involved in polysaccharide biosynthesis and the structure of the polysaccharide (LPS or capsule). This will facilitate an initial prediction of the gene function and the design of the biochemical experiments to analyze enzyme–substrate reactions. Second, to obtain unequivocal data of the function of polysaccharide genes, one needs to overexpress and purify the proteins encoded by the genes. Third, the purified proteins will then be used in enzyme–substrate reactions to resolve their biochemical functions. Fourth, the approach of using CE-MS/MS and NMR provides exquisite resolution of substrates and products and the ultimate identification of the chemical structure of the reaction products.

5.3. Biosynthesis of UDP-*N*-Acetyl-D-Quinovanosamine

The biochemical characterization of the function of WbpM was facilitated by overexpression of WbpM with a N-terminal histidine-tag (His-WbpM). WbpM consists of 665 amino acids with a transmembrane domain at the amino terminus. The recombinant form of His-WbpM expressed in *E. coli* is targeted at the inner membrane. SDS-PAGE analysis revealed that His-WbpM migrated 13 kDa smaller than its predicted size. A His-WbpM truncated version was generated with the N-terminal 131 amino acids removed and this truncated protein migrated in SDS-PAGE with the expected molecular mass. This observation demonstrated that the membrane-bound portion caused an anomalous migration observed on SDS-PAGE. The localization of WbpM within the inner membrane was investigated using reporter proteins fused to various regions within the C-terminus of His-WbpM. The reporter proteins used were alkaline phosphatase and β -galactosidase and these markers identify the localization within either the periplasmic space or the cytoplasm, respectively¹¹⁹. Based on these techniques, a topology model of WbpM was proposed and four putative transmembrane domains were localized at amino acid positions 20–41, 51–73, 84–106 and 117–134, respectively. Both the N-terminus and the carboxyl domain of WbpM are located on the cytoplasmic face of the inner membrane. Another intriguing observation made in the same study was from complementation of a *wbpM* mutant with the truncated version of *wbpM* lacking the transmembrane region of the gene.

LPS from the complemented strain showed a change in the modal distribution of the B-band LPS banding patterns in SDS-PAGE analysis as well as the lack of the high-molecular-weight portion of the LPS. These results showed that although the transmembrane domains of WbpM were not critical for enzymatic activity, this region of the protein plays a role in modulating the degree of polymerization of high-molecular-weight B-band LPS in vivo.

Biochemical characterization of the full-length form of His-WbpM in membrane fractions was performed³⁶. Complete conversion of UDP-D-GlcNAc to product was achieved under an optimal pH of 10. The predicted product, UDP-D-QuiNAc is not commercially available. Fortunately, we were able to produce UDP-D-QuiNAc by a previously characterized reaction using FlaA1 of *H. pylori*, which is a C-6 dehydratase that converts UDP-D-GlcNAc to UDP-D-QuiNAc^{36, 37}. Interestingly, FlaA1 is homologous to the C-terminus of the WbpM protein and does not contain the N-terminus transmembrane domains. Our results from CE-MS/MS analysis showed that WbpM (either the native form or a His-tagged form) was capable of utilizing UDP-D-GlcNAc as a substrate and produced the same product as FlaA1^{36, 37}. These results substantiated the data obtained from complementation studies, that WbpM and FlaA1 are functional homologues of each other and possess C-6 dehydratase activity.

For determination of kinetic parameters, a soluble form of WbpM was required. Five truncated versions of WbpM (His-L133, His-R182, His-S234, His-S262 and His-V285) were constructed because full-length His-WbpM solubilized with Triton X-100 was inactive. Only one of the constructs, His-S262, was found to be soluble and could be purified to homogeneity for characterization. The kinetic parameters (K_m , k_{cat}) determined under optimal conditions, pH 10, 30°C in the presence of NAD, are shown in Table 1. CE-MS analysis of the His-S262 reaction product confirmed that indeed a C-6 deoxyderivative of UDP-D-GlcNAc was produced by the enzyme-substrate reaction³⁶. The WbpM His-S262 version used specifically UDP-D-GlcNAc as a substrate and no reaction products using other substrates including UDP-D-Glc, UDP-D-GalNAc, UDP-D-Gal, GDP-D-mannose, dTDP-D-Glc and CDP-D-Glc were observed.

Proteins that belong to the SDR family have at the active site a serine (S), tyrosine (Y) and lysine (K) residues which form the catalytic triad⁸⁶. In FlaA1, WbjC and Cap5F, the catalytic triad consists of SYK, in contrast, WbpM, WbjB and Cap5E have SMK in which the Y is replaced with M (Met). The role of the central tyrosine and methionine in the catalytic mechanisms of FlaA1 and WbpM was studied by site-directed mutagenesis³⁸. A FlaA1 Y141F mutant was totally inactive, indicating an essential role of the hydroxyl side group of the tyrosine residue for catalysis³⁸. To study the role of Y vs M, a FlaA1 Y141M mutant was constructed, such that the SYK was replaced with

SMK as the catalytic triad. This mutant was moderately active and exhibited a pH optimum profile similar to WbpM³⁸. The secondary structure of the FlaA1 Y141M mutant at pH 5 and pH 9 were analyzed by using circular dichroism and no significant secondary structure changes were observed. Thus, the pH optimum change in the FlaA1 mutant was not due to structural change of the protein. These data, suggested that the Tyr residue plays a role in governing the pH of the active site. The reverse experiment was performed to introduce a SYK catalytic triad in WbpM; this mutant, WbpM M438Y was shown to be active. The substrate and product specificities of these two catalytic mutants (Y141M in FlaA1 and M438Y in WbpM) were the same as the FlaA1 and WbpM (Creuzenet *et al.*³⁸). Our results indicate that the catalytic triad can be either SYK or SMK.

Understanding the enzymes involved in biochemical pathways of unique nucleotide sugar biosynthesis provide the fundamental knowledge toward a better understanding of O-antigen synthesis and assembly. The approach used in these analyses provides exquisite resolution and unequivocal identification of structures of the products from enzyme–substrate reactions. The data from these studies are invaluable for in-depth structure–function studies involving X-ray crystallography to distinguish substrate specificity and regions of the protein that dictates whether an enzyme is an epimerase, a reductase or a dehydratase.

6. CONCLUSIONS AND FUTURE DIRECTIONS

The fact that different modifications can occur in lipid A from wild-type strains vs clinical isolates of *P. aeruginosa* provides the evidence of adaptability of this bacterium in various environments. The presence of A-band O antigen, B-band O antigen and the diversity afforded by subtle changes in the structures of B-band among the serotypes further demonstrate the remarkable diversity in surface polysaccharides exhibited by this bacterial species. To date, we feel privileged to know the organization of the biosynthetic gene clusters from all 20 serotypes as well as the structural data of the O antigens. This information provides the foundation whereby we can systematically solve individual biosynthetic pathways for the production of a number of very interesting and rare sugars that are essential for the production of surface polysaccharides in *P. aeruginosa* and other bacterial pathogens.

The information from structural elucidation of the two core OS glycoforms and whole genome sequence annotation are presently our most valuable tools for further investigation into the mechanisms of core OS biosynthesis in *P. aeruginosa*. A number of questions that need to be addressed in future research include (a) What are the mechanisms of phosphorylation of the inner core? (b) How is core OS regulated by changes in the environment? Does the

rhamnosyltransferase, MigA, play a role in this regulation? (c) Where are the genes that encode the rest of the glucosyltransferases in the *P. aeruginosa* genome? It would be interesting to determine if the ORFs of unknown function at the 3' end of the *waaF* operon encode the three glucosyltransferases required for synthesis of the outer-core OS because it would lead to the identification of a novel family of glycosyltransferases.

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REFERENCES

1. Akatova, N.S. and Smirnova, N.E., 1982, Serological classification of *Pseudomonas aeruginosa*. *Zh. Mikrobiol. Epidemiol. Immunobiol.*, 7:87–91.
2. Alexander, D.C. and Valvano, M.A., 1994, Role of the *rfe* gene in the biosynthesis of the *Escherichia coli* O7-specific lipopolysaccharide and other O-specific polysaccharides containing *N*-acetylglucosamine. *J. Bacteriol.*, 176:7079–7084.
3. Alpern, E.R., Alessandrini, E.A., McGowan, K.L., Bell, L.M., and Shaw, K.N., 2001, Serotype prevalence of occult pneumococcal bacteremia. *Pediatrics*, 108:E23.
4. Amer, A.O. and Valvano, M.A., 2001, Conserved amino acid residues found in a predicted cytosolic domain of the lipopolysaccharide biosynthetic protein WecA are implicated in the recognition of UDP-*N*-acetylglucosamine. *Microbiology*, 147:3015–3025.
5. Amer, A.O. and Valvano, M.A., 2002, Conserved aspartic acids are essential for the enzymic activity of the WecA protein initiating the biosynthesis of O-specific lipopolysaccharide and enterobacterial common antigen in *Escherichia coli*. *Microbiology*, 148:571–582.
6. Amor, P.A. and Whitfield, C., 1997, Molecular and functional analysis of genes required for expression of group IB K antigens in *Escherichia coli*: Characterization of the *his*-region containing gene clusters for multiple cell-surface polysaccharides. *Mol. Microbiol.*, 26:145–161.
7. Anderson, M.S., Robertson, A.D., Macher, I., and Raetz, C.R., 1988, Biosynthesis of lipid A in *Escherichia coli*: Identification of UDP-3-*O*-[(*R*)-3-hydroxymyristoyl]- α -D-glucosamine as a precursor of UDP-N₂O₃-bis[(*R*)-3-hydroxymyristoyl]- α -D-glucosamine. *Biochemistry*, 27:1908–1917.
8. Babinski, K.J., Ribeiro, A.A., and Raetz, C.R., 2002, The *Escherichia coli* gene encoding the UDP-2,3-diacetylglucosamine pyrophosphatase of lipid A biosynthesis. *J. Biol. Chem.*, 277:25937–25946.
9. Bailey, M.J., Hughes, C., and Koronakis, V., 2000, *In vitro* recruitment of the RfaH regulatory protein into a specialised transcription complex, directed by the nucleic acid *ops* element. *Mol. Gen. Genet.*, 262:1052–1059.

10. Bailey, M.J., Hughes, C., and Koronakis, V., 1997, RfaH and the *ops* element, components of a novel system controlling bacterial transcription elongation. *Mol. Microbiol.*, 26:845–851.
11. Bastin, D.A., Stevenson, G., Brown, P.K., Haase, A., and Reeves, P.R., 1993, Repeat unit polysaccharides of bacteria: A model for polymerization resembling that of ribosomes and fatty acid synthetase, with a novel mechanism for determining chain length. *Mol. Microbiol.*, 7:725–734.
12. Batchelor, R.A., Alifano, P., Biffali, E., Hull, S.I., and Hull, R.A., 1992, Nucleotide sequences of the genes regulating O-polysaccharide antigen chain length (*rol*) from *Escherichia coli* and *Salmonella typhimurium*: Protein homology and functional complementation. *J. Bacteriol.*, 174:5228–5236.
13. Bateman, A., Birney, E., Cerruti, L., Durbin, R., Eddy, S.R., Griffiths-Jones, S., Howe, K.L., Marshall, M., and Sonnhammer, E.L., 2002, The Pfam protein families database. *Nucleic Acids Res.*, 30:276–280.
14. Bateman, A., Birney, E., Durbin, R., Eddy, S.R., Howe, K.L., and Sonnhammer, E.L., 2000, The Pfam protein families database. *Nucleic Acids Res.*, 28:263–266.
15. Beall, B. and Lutkenhaus, J., 1987, Sequence analysis, transcriptional organization, and insertional mutagenesis of the *envA* gene of *Escherichia coli*. *J. Bacteriol.*, 169:5408–5415.
16. Beckmann, F., Moll, H., Jäger, K.E., and Zähringer, U., 1995, Preliminary communication 7-O-carbamoyl-L-glycero-D-manno-heptose: A new core constituent in the lipopolysaccharide of *Pseudomonas aeruginosa*. *Carbohydr. Res.*, 267:C3–C7.
17. Bélanger, M., Burrows, L.L., and Lam, J.S., 1999, Functional analysis of genes responsible for the synthesis of the B-band O antigen of *Pseudomonas aeruginosa* serotype O6 lipopolysaccharide. *Microbiology*, 145:3505–3521.
18. Bert, F. and Lambert-Zechovsky, N., 1996, Comparative distribution of resistance patterns and serotypes in *Pseudomonas aeruginosa* isolates from intensive care units and other wards. *J. Antimicrob. Chemother.*, 37:809–813.
19. Beveridge, T.J., Makin, S.A., Kadurugamuwa, J.L., and Li, Z., 1997, Interactions between biofilms and the environment. *FEMS Microbiol. Rev.*, 20:291–303.
20. Bhat, R., Marx, A., Galanos, C., and Conrad, R.S., 1990, Structural studies of lipid A from *Pseudomonas aeruginosa* PAO1: Occurrence of 4-amino-4-deoxyarabinose. *J. Bacteriol.*, 172:6631–6636.
21. Blankenfheldt, W., Asuncion, M., Lam, J.S., and Naismith, J.H., 2000, The structural basis of the catalytic mechanism and regulation of glucose-1-phosphate thymidyltransferase (RmlA). *EMBO J.*, 19:6652–6663.
22. Blankenfheldt, W., Giraud, M.F., Leonard, G., Rahim, R., Creuzenet, C., Lam, J.S., and Naismith, J.H., 2000, The purification, crystallization and preliminary structural characterization of glucose-1-phosphate thymidyltransferase (RmlA), the first enzyme of the dTDP-L-rhamnose synthesis pathway from *Pseudomonas aeruginosa*. *Acta Crystallogr. D. Biol. Crystallogr.*, 56:1501–1504.
23. Brodsky, I.E., Ernst, R.K., Miller, S.I., and Falkow, S., 2002, *mig-14* is a *Salmonella* gene that plays a role in bacterial resistance to antimicrobial peptides. *J. Bacteriol.*, 184:3203–3213.
24. Brozek, K.A. and Raetz, C.R., 1990, Biosynthesis of lipid A in *Escherichia coli*. Acyl carrier protein-dependent incorporation of laurate and myristate. *J. Biol. Chem.*, 265:15410–15417.
25. Bunnell, E., Lynn, M., Habet, K., Neumann, A., Perdomo, C.A., Friedhoff, L.T., Rogers, S.L., and Parrillo, J.E., 2000, A lipid A analog, E5531, blocks the endotoxin response in human volunteers with experimental endotoxemia. *Crit. Care Med.*, 28:2713–2720.
26. Burrows, L.L., Charter, D.F., and Lam, J.S., 1996, Molecular characterization of the *Pseudomonas aeruginosa* serotype O5 (PAO1) B-band lipopolysaccharide gene cluster. *Mol. Microbiol.*, 22:481–495.
27. Burrows, L.L., Chow, D., and Lam, J.S., 1997, *Pseudomonas aeruginosa* B-band O-antigen chain length is modulated by Wzz (Ro1). *J. Bacteriol.*, 179:1482–1489.

28. Burrows, L.L. and Lam, J.S., 1999, Effect of *wzx* (*rfbX*) mutations on A-band and B-band lipopolysaccharide biosynthesis in *Pseudomonas aeruginosa* O5. *J. Bacteriol.*, 181:973–980.
29. Burrows, L.L., Urbanic, R.V., and Lam, J.S., 2000, Functional conservation of the polysaccharide biosynthetic protein WbpM and its homologues in *Pseudomonas aeruginosa* and other medically significant bacteria. *Infect. Immun.*, 68:931–936.
30. Bystrova, O.V., Shashkov, A.S., Kocharova, N.A., Knirel, Y.A., Lindner, B., Zähringer, U., and Pier, G.B., 2002, Structural studies on the core and the O-polysaccharide repeating unit of *Pseudomonas aeruginosa* immunotype 1 lipopolysaccharide. *Eur. J. Biochem.*, 269:2194–2203.
31. Chen, M.H., Steiner, M.G., de Laszlo, S.E., Patchett, A.A., Anderson, M.S., Hyland, S.A., Onishi, H.R., Silver, L.L., and Raetz, C.R., 1999, Carbohydroxamido-oxazolidines: Antibacterial agents that target lipid A biosynthesis. *Bioorg. Med. Chem. Lett.*, 9:313–318.
32. Clementz, T., Bednarski, J.J., and Raetz, C.R., 1996, Function of the *htrB* high temperature requirement gene of *Escherichia coli* in the acylation of lipid A: HtrB catalyzed incorporation of laurate. *J. Biol. Chem.*, 271:12095–12102.
33. Coimbra, R.S., Grimont, F., and Grimont, P.A., 1999, Identification of *Shigella* serotypes by restriction of amplified O-antigen gene cluster. *Res. Microbiol.*, 150:543–553.
34. Coyne, M.J., Jr., Russell, K.S., Coyle, C.L., and Goldberg, J.B., 1994, The *Pseudomonas aeruginosa* *algC* gene encodes phosphoglucomutase, required for the synthesis of a complete lipopolysaccharide core. *J. Bacteriol.*, 176:3500–3507.
35. Creuzenet, C., Bélanger, M., Wakarchuk, W.W., and Lam, J.S., 2000, Expression, purification, and biochemical characterization of WbpP, a new UDP-GlcNAc C4 epimerase from *Pseudomonas aeruginosa* serotype O6. *J. Biol. Chem.*, 275:19060–19067.
36. Creuzenet, C. and Lam, J.S., 2001, Topological and functional characterization of WbpM, an inner membrane UDP-GlcNAc C6 dehydratase essential for lipopolysaccharide biosynthesis in *Pseudomonas aeruginosa*. *Mol. Microbiol.*, 41:1295–1310.
37. Creuzenet, C., Schur, M.J., Li, J., Wakarchuk, W.W., and Lam, J.S., 2000, FlaA1, a new bifunctional UDP-GlcNAc C6 Dehydratase/ C4 reductase from *Helicobacter pylori*. *J. Biol. Chem.*, 275:34873–34880.
38. Creuzenet, C., Urbanic, R.V., and Lam, J.S., 2002, Structure-function studies of two novel UDP-GlcNAc C6 dehydratases/C4 reductases. Variation from the SYK dogma. *J. Biol. Chem.*, 277:26769–26778.
39. Daniels, C., Griffiths, C., Cowles, B., and Lam, J.S., 2002, *Pseudomonas aeruginosa* O-antigen chain length is determined before ligation to lipid A core. *Environ. Microbiol.*, 4:883–897.
40. Daniels, C., Vindurampulle, C., and Morona, R., 1998, Overexpression and topology of the *Shigella flexneri* O-antigen polymerase (Rfc/Wzy). *Mol. Microbiol.*, 28:1211–1222.
41. de Kievit, T.R., Dasgupta, T., Schweizer, H., and Lam, J.S., 1995, Molecular cloning and characterization of the *rfc* gene of *Pseudomonas aeruginosa* (serotype O5). *Mol. Microbiol.*, 16:565–574.
42. de Kievit, T.R. and Lam, J.S., 1997, Isolation and characterization of two genes, *waaC* (*rfaC*) and *waaF* (*rfaF*), involved in *Pseudomonas aeruginosa* serotype O5 inner-core biosynthesis. *J. Bacteriol.*, 179:3451–3457.
43. de Kievit, T.R. and Lam, J.S., 1994, Monoclonal antibodies that distinguish inner core, outer core, and lipid A regions of *Pseudomonas aeruginosa* lipopolysaccharide. *J. Bacteriol.*, 176:7129–7139.
44. de Kievit, T.R., Staples, T., and Lam, J.S., 1997, *Pseudomonas aeruginosa* *rfc* genes of serotypes O2 and O5 could complement O-polymerase-deficient semi-rough mutants of either serotype. *FEMS Microbiol. Lett.*, 147:251–257.

45. Dean, C.R., Datta, A., Carlson, R.W., and Goldberg, J.B., 2002, WbjA adds glucose to complete the O-antigen trisaccharide repeating unit of the lipopolysaccharide of *Pseudomonas aeruginosa* serogroup O11. *J. Bacteriol.*, 184:323–326.
46. Dean, C.R., Franklund, C.V., Retief, J.D., Coyne, M.J., Jr., Hatano, K., Evans, D.J., Pier, G.B., and Goldberg, J.B., 1999, Characterization of the serogroup O11 O-antigen locus of *Pseudomonas aeruginosa* PA103. *J. Bacteriol.*, 181:4275–4284.
47. Dean, C.R. and Goldberg, J.B., 2002, *Pseudomonas aeruginosa* galU is required for a complete lipopolysaccharide core and repairs a secondary mutation in a PA103 (serogroup O11) wbpM mutant. *FEMS Microbiol. Lett.*, 210:277–283.
48. Dean, C.R. and Goldberg, J.B., 2000, The wbpM gene in *Pseudomonas aeruginosa* serogroup O17 resides on a cryptic copy of the serogroup O11 O antigen gene locus. *FEMS Microbiol. Lett.*, 187:59–63.
49. deLancey Pulcini, E. and Camper, A., 2002, Proteomic analysis of variations in protein expression in *Pseudomonas aeruginosa* during initial adhesion. Abstr. Q-256. In *Abstracts of the 102nd General Meeting of the American Society for Microbiology 2002*. American Society for Microbiology, Washington, DC.
50. DiGiandomenico, A., Mawish, M.J., Bisailon, A., Stehle, J.R., Lam, J.S., and Castric, P., 2002, Glycosylation of *Pseudomonas aeruginosa* 1244 pilin: Glycan substrate specificity. *Mol. Microbiol.*, 46:519–530.
51. Doerrler, W.T. and Raetz, C.R., 2002, ATPase activity of the MsbA lipid flippase of *Escherichia coli*. *J. Biol. Chem.*, 277:36697–36705.
52. Doerrler, W.T., Reedy, M.C., and Raetz, C.R., 2001, An *Escherichia coli* mutant defective in lipid export. *J. Biol. Chem.*, 276:11461–11464.
53. Dotson, G.D., Kaltashov, I.A., Cotter, R.J., and Raetz, C.R., 1998, Expression cloning of a *Pseudomonas* gene encoding a hydroxydecanoyl-acyl carrier protein-dependent UDP-GlcNAc acyltransferase. *J. Bacteriol.*, 180:330–337.
54. Drewry, D.T., Symes, K.C., Gray, G.W., and Wilkinson, S.G., 1975, Studies of polysaccharide fractions from the lipopolysaccharide of *Pseudomonas aeruginosa* N.C.T.C. 1999. *Biochem. J.*, 149:93–106.
55. Eagon, R.G., Simmons, G.P., and Carson, K.J., 1965, Evidence for the presence of ash and fivalent metals in the cell wall of *Pseudomonas aeruginosa*. *Can. J. Microbiol.*, 11:1041–1042.
56. Ernst, R.K., Yi, E.C., Guo, L., Lim, K.B., Burns, J.L., Hackett, M., and Miller, S.I., 1999, Specific lipopolysaccharide found in cystic fibrosis airway *Pseudomonas aeruginosa*. *Science*, 286:1561–1565.
57. Farmer, J.J., 3rd, Weinstein, R.A., Zierdt, C.H., and Brokopp, C.D., 1982, Hospital outbreaks caused by *Pseudomonas aeruginosa*: Importance of serogroup O11. *J. Clin. Microbiol.*, 16:266–270.
58. Fisher, M.W., Devlin, H.B., and Gnabasik, F.J., 1969, New immunotype schema for *Pseudomonas aeruginosa* based on protective antigens. *J. Bacteriol.*, 98:835–836.
59. Fomsgaard, A., Høiby, N., Shand, G.H., Conrad, R.S., and Galanos, C., 1988, Longitudinal study of antibody response to lipopolysaccharides during chronic *Pseudomonas aeruginosa* lung infection in cystic fibrosis. *Infect. Immun.*, 56:2270–2278.
60. Fournier, J.M., Vann, W.F., and Karakawa, W.W., 1984, Purification and characterization of *Staphylococcus aureus* type 8 capsular polysaccharide. *Infect. Immun.*, 45:87–93.
61. Garrett, T.A., Kadmas, J.L., and Raetz, C.R., 1997, Identification of the gene encoding the *Escherichia coli* lipid A 4'-kinase. Facile phosphorylation of endotoxin analogs with recombinant LpxK. *J. Biol. Chem.*, 272:21855–21864.
62. Giraud, M.F. and Naismith, J.H., 2000, The rhamnose pathway. *Curr. Opin. Struct. Biol.*, 10:687–696.

63. Giwercman, B., Fomsgaard, A., Mansa, B., and Høiby, N., 1992, Polyacrylamide gel electrophoresis analysis of lipopolysaccharide from *Pseudomonas aeruginosa* growing planktonically and as biofilm. *FEMS Microbiol. Immunol.*, 4:225–229.
64. Goldberg, J.B., Hatano, K., and Pier, G.B., 1993, Synthesis of lipopolysaccharide O side chains by *Pseudomonas aeruginosa* PAO1 requires the enzyme phosphomannomutase. *J. Bacteriol.*, 175:1605–1611.
65. Goldman, R., Kohlbrenner, W., Lartey, P., and Pernet, A., 1987, Antibacterial agents specifically inhibiting lipopolysaccharide synthesis. *Nature*, 329:162–164.
66. Goldman, R.C., Doran, C.C., Kadam, S.K., and Capobianco, J.O., 1988, Lipid A precursor from *Pseudomonas aeruginosa* is completely acylated prior to addition of 3-deoxy-D-manno-octulosonate. *J. Biol. Chem.*, 263:5217–5223.
67. Graninger, M., Nidetzky, B., Heinrichs, D.E., Whitfield, C., and Messner, P., 1999, Characterization of dTDP-4-dehydroxymannose 3,5-epimerase and dTDP-4-dehydroxymannose reductase, required for dTDP-L-rhamnose biosynthesis in *Salmonella enterica* serovar Typhimurium LT2. *J. Biol. Chem.*, 274:25069–25077.
68. Gray, G.W. and Wilkinson, S.G., 1965, The effect of ethylenediaminetetra-acetic acid on the cell walls of some gram-negative bacteria. *J. Gen. Microbiol.*, 39:385–399.
69. Groisman, E.A., 2001, The pleiotropic two-component regulatory system PhoP-PhoQ. *J. Bacteriol.*, 183:1835–1842.
70. Habs, I., 1957, Untersuchungen über die O-Antigene von *Pseudomonas aeruginosa*. *Z. Hyg. Infekt.-Kr.*, 144:218–228.
71. Hammond, S.M., Claesson, A., Jansson, A.M., Larsson, L.G., Pring, B.G., Town, C.M., and Ekstrom, B., 1987, A new class of synthetic antibacterials acting on lipopolysaccharide biosynthesis. *Nature*, 327:730–732.
72. Hancock, R.E., Mutharia, L.M., Chan, L., Darveau, R.P., Speert, D.P., and Pier, G.B., 1983, *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis: A class of serum-sensitive, nontypable strains deficient in lipopolysaccharide O side chains. *Infect. Immun.*, 42:170–177.
73. Haseley, S.R., Holst, O., and Brade, H., 1997, Structural and serological characterisation of the O-antigenic polysaccharide of the lipopolysaccharide from *Acinetobacter haemolyticus* strain ATCC 17906. *Eur. J. Biochem.*, 244:761–766.
74. Hashimoto, Y., Li, N., Yokoyama, H., and Ezaki, T., 1993, Complete nucleotide sequence and molecular characterization of *ViaB* region encoding Vi antigen in *Salmonella typhi*. *J. Bacteriol.*, 175:4456–4465.
75. Heinrichs, D.E., Monteiro, M.A., Perry, M.B., and Whitfield, C., 1998, The assembly system for the lipopolysaccharide R2 core-type of *Escherichia coli* is a hybrid of those found in *Escherichia coli* K-12 and *Salmonella enterica*. Structure and function of the R2 WaaK and WaaL homologs. *J. Biol. Chem.*, 273:8849–8859.
76. Hobbs, M. and Reeves, P.R., 1994, The JUMPstart sequence: A 39 bp element common to several polysaccharide gene clusters. *Mol. Microbiol.*, 12:855–856.
77. Homma, Y.J., 1976, A new antigenic scheme and live cell slide agglutination procedure for the intrasubspecific, serologic classification of *Pseudomonas aeruginosa*. *Jap. J. Exp. Med.*, 46:329–336.
78. Hyland, S.A., Eveland, S.S., and Anderson, M.S., 1997, Cloning, expression, and purification of UDP-3-O-acyl-GlcNAc deacetylase from *Pseudomonas aeruginosa*: A metalloamidase of the lipid A biosynthesis pathway. *J. Bacteriol.*, 179:2029–2037.
79. Jackman, J.E., Fierke, C.A., Tumey, L.N., Pirrung, M., Uchiyama, T., Tahir, S.H., Hindsgaul, O., and Raetz, C.R., 2000, Antibacterial agents that target lipid A biosynthesis in gram-negative bacteria. Inhibition of diverse UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine

- deacetylases by substrate analogs containing zinc binding motifs. *J. Biol. Chem.*, 275:11002–11009.
80. Jackman, J.E., Raetz, C.R., and Fierke, C.A., 2001, Site-directed mutagenesis of the bacterial metalloamidase UDP-(3-*O*-acyl)-*N*-acetylglucosamine deacetylase (LpxC). Identification of the zinc binding site. *Biochemistry*, 40:514–523.
 81. Jackman, J.E., Raetz, C.R., and Fierke, C.A., 1999, UDP-3-*O*-(*R*-3-hydroxymyristoyl)-*N*-acetylglucosamine deacetylase of *Escherichia coli* is a zinc metalloenzyme. *Biochemistry*, 38:1902–1911.
 82. Jiang, S.M., Wang, L., and Reeves, P.R., 2001, Molecular characterization of *Streptococcus pneumoniae* type 4, 6B, 8, and 18C capsular polysaccharide gene clusters. *Infect. Immun.*, 69:1244–1255.
 83. Jones, C., Currie, F., and Forster, M.J., 1991, N.m.r. and conformational analysis of the capsular polysaccharide from *Streptococcus pneumoniae* type 4. *Carbohydr. Res.*, 221:95–121.
 84. Jörnvall, H., 1999, Multiplicity and complexity of SDR and MDR enzymes. *Adv. Exp. Med. Biol.*, 463:359–364.
 85. Jörnvall, H., Hoog, J.O., and Persson, B., 1999, SDR and MDR: Completed genome sequences show these protein families to be large, of old origin, and of complex nature. *FEBS Lett.*, 445:261–264.
 86. Kallberg, Y., Oppermann, U., Jörnvall, H. and Persson, B., 2002, Short-chain dehydrogenases/reductases (SDRs). *Eur. J. Biochem.*, 269:4409–4417.
 87. Karunaratne, D.N., Richards, J.C., and Hancock, R.E., 1992, Characterization of lipid A from *Pseudomonas aeruginosa* O-antigenic B band lipopolysaccharide by 1D and 2D NMR and mass spectral analysis. *Arch. Biochem. Biophys.*, 299:368–376.
 88. Kasper, D.L., Weintraub, A., Lindberg, A.A., and Lonngren, J., 1983, Capsular polysaccharides and lipopolysaccharides from two *Bacteroides fragilis* reference strains: Chemical and immunochemical characterization. *J. Bacteriol.*, 153:991–997.
 89. Kelly, T.M., Stachula, S.A., Raetz, C.R., and Anderson, M.S., 1993, The *firA* gene of *Escherichia coli* encodes UDP-3-*O*-(*R*-3-hydroxymyristoyl)-glucosamine *N*-acyltransferase. The third step of endotoxin biosynthesis. *J. Biol. Chem.*, 268:19866–19874.
 90. Kline, T., Andersen, N.H., Harwood, E.A., Bowman, J., Malanda, A., Endsley, S., Erwin, A.L., Doyle, M., Fong, S., Harris, A.L., Mendelsohn, B., Mdluli, K., Raetz, C.R., Stover, C.K., Witte, P.R., Yabannavar, A., and Zhu, S., 2002, Potent, novel *in vitro* inhibitors of the *Pseudomonas aeruginosa* deacetylase LpxC. *J. Med. Chem.*, 45:3112–3129.
 91. Kneidinger, B., Larocque, S., Brisson, J.R., Cadotte, N., and Lam, J.S., 2003, Biosynthesis of 2-acetamido-2,6-dideoxy-L-hexoses in bacteria follows a pattern distinct from those of the pathways of 6-deoxy-L-hexoses. *Biochem. J.*, 371:989–995.
 92. Kneidinger, B., Marolda, C., Graninger, M., Zamyatina, A., McArthur, F., Kosma, P., Valvano, M.A., and Messner, P., 2002, Biosynthesis pathway of ADP-L-glycero- β -D-manno-heptose in *Escherichia coli*. *J. Bacteriol.*, 184:363–369.
 93. Kneidinger, B., O’Riordan, K., Li, J., Brisson, J.R., Lee, J.C., and Lam, J.S., 2003, Three highly conserved proteins catalyze the conversion of UDP-*N*-acetyl-D-glucosamine to precursors for the biosynthesis of O antigen in *Pseudomonas aeruginosa* O11 and capsule in *Staphylococcus aureus* type 5. Implications for the UDP-*N*-acetyl-L-fucosamine biosynthetic pathway. *J. Biol. Chem.*, 278:3615–3627.
 94. Knirel, Y.A., 1990, Polysaccharide antigens of *Pseudomonas aeruginosa*. *Crit. Rev. Microbiol.*, 17:273–304.
 95. Knirel, Y.A., Bystrova, O.V., Shashkov, A.S., Lindner, B., Kocharova, N.A., Senchenkova, S.N., Moll, H., Zähringer, U., Hatano, K., and Pier, G.B., 2001, Structural analysis of the lipopolysaccharide core of a rough, cystic fibrosis isolate of *Pseudomonas aeruginosa*. *Eur. J. Biochem.*, 268:4708–4719.

96. Knirel, Y.A., Helbig, J.H., and Zähringer, U., 1996, Structure of a decasaccharide isolated by mild acid degradation and dephosphorylation of the lipopolysaccharide of *Pseudomonas fluorescens* strain ATCC 49271. *Carbohydr. Res.*, 283:129–139.
97. Knirel, Y.A. and Kochetkov, N.K., 1994, The structure of lipopolysaccharides of gram-negative bacteria. III. The structure of O-antigens: A review. *Biochemistry*, 59:1325–1382.
98. Knirel, Y.A., Vinogradov, E.V., Kocharova, N.A., Paramonov, N.A., Kochetkov, N.K., Dmitriev, B.A., Stanislavsky, E.S., and Lanyi, B., 1988, The structure of O-specific polysaccharides and serological classification of *Pseudomonas aeruginosa* (a review). *Acta Microbiol. Hung.*, 35:3–24.
99. Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E.L., 2001, Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. *J. Mol. Biol.*, 305:567–580.
100. Kulshin, V.A., Zähringer, U., Lindner, B., Jäger, K.E., Dmitriev, B.A., and Rietschel, E.T., 1991, Structural characterization of the lipid A component of *Pseudomonas aeruginosa* wild-type and rough mutant lipopolysaccharides. *Eur. J. Biochem.*, 198:697–704.
101. Kuzio, J. and Kropinski, A.M., 1983, O-antigen conversion in *Pseudomonas aeruginosa* PAO1 by bacteriophage D3. *J. Bacteriol.*, 155:203–212.
102. Lam, J.S., Handelsman, M.Y., Chivers, T.R., and MacDonald, L.A., 1992, Monoclonal antibodies as probes to examine serotype-specific and cross-reactive epitopes of lipopolysaccharides from serotypes O2, O5, and O16 of *Pseudomonas aeruginosa*. *J. Bacteriol.*, 174:2178–2184.
103. Lam, J.S., MacDonald, L.A., Kropinski, A.M., and Speert, D.P., 1988, Characterization of non-typable strains of *Pseudomonas aeruginosa* from cystic fibrosis patients by means of monoclonal antibodies and SDS-polyacrylamide gel electrophoresis. *Serodiagn. Immunother. Infect. Dis.*, 2:365–374.
104. Lam, J.S., MacDonald, L.A., and Lam, M.Y., 1987, Production of monoclonal antibodies against serotype strains of *Pseudomonas aeruginosa*. *Infect. Immun.*, 55:2854–2856.
105. Lam, J.S., MacDonald, L.A., Lam, M.Y., Duchesne, L.G., and Southam, G.G., 1987, Production and characterization of monoclonal antibodies against serotype strains of *Pseudomonas aeruginosa*. *Infect. Immun.*, 55:1051–1057.
106. Lamping, N., Hoess, A., Yu, B., Park, T.C., Kirschning, C.J., Pfeil, D., Reuter, D., Wright, S.D., Herrmann, F., and Schumann, R.R., 1996, Effects of site-directed mutagenesis of basic residues (Arg 94, Lys 95, Lys 99) of lipopolysaccharide (LPS)-binding protein on binding and transfer of LPS and subsequent immune cell activation. *J. Immunol.*, 157:4648–4656.
107. Lanyi, B., 1966, Serological properties of *Pseudomonas aeruginosa*. I. Group-specific somatic antigens. *Acta Microbiol. Acad. Sci. Hung.*, 13:295–318.
108. Lee, J.C. and Lee, C.Y., 1999, Capsular polysaccharides of *Staphylococcus aureus*. In J.B. Goldberg (ed.), *Genetics of Bacterial Polysaccharides*, pp. 185–205. CRC Press, Boca Raton, FL.
109. Lesley, J.A. and Waldburger, C.D., 2001, Comparison of the *Pseudomonas aeruginosa* and *Escherichia coli* PhoQ sensor domains: Evidence for distinct mechanisms of signal detection. *J. Biol. Chem.*, 276:30827–30833.
110. Li, X., Uchiyama, T., Raetz, C.R., and Hindsgaul, O., 2003, Synthesis of a Carbohydrate-derived hydroxamic acid inhibitor of the bacterial enzyme (LpxC) involved in lipid A biosynthesis. *Org. Lett.*, 5:539–541.
111. Lin, W.S., Cunneen, T., and Lee, C.Y., 1994, Sequence analysis and molecular characterization of genes required for the biosynthesis of type 1 capsular polysaccharide in *Staphylococcus aureus*. *J. Bacteriol.*, 176:7005–7016.
112. Liu, D., Cole, R.A., and Reeves, P.R., 1996, An O-antigen processing function for Wzx (RfbX): A promising candidate for O-unit flippase. *J. Bacteriol.*, 178:2102–2107.

113. Liu, P.V., Matsomoto, H., Kusama, H., and Bergan, T., 1983, Survey of heat-stable major somatic antigens of *Pseudomonas aeruginosa*. *Int. J. Syst. Bacteriol.*, 33:256–275.
114. Liu, P.V. and Wang, S., 1990, Three new major somatic antigens of *Pseudomonas aeruginosa*. *J. Clin. Microbiol.*, 28:922–925.
115. Lynn, W.A. and Golenbock, D.T., 1992, Lipopolysaccharide antagonists. *Immunol. Today*, 13:271–276.
116. Macfarlane, E.L., Kwasnicka, A., Ochs, M.M., and Hancock, R.E., 1999, PhoP-PhoQ homologues in *Pseudomonas aeruginosa* regulate expression of the outer-membrane protein OprH and polymyxin B resistance. *Mol. Microbiol.*, 34:305–316.
117. MacIntyre, S., Lucken, R., and Owen, P., 1986, Smooth lipopolysaccharide is the major protective antigen for mice in the surface extract from IATS serotype 6 contributing to the polyvalent *Pseudomonas aeruginosa* vaccine PEV. *Infect. Immun.*, 52:76–84.
118. Manca, M.C., Weintraub, A., and Widmalm, G., 1996, Structural studies of the *Escherichia coli* O26 O-antigen polysaccharide. *Carbohydr. Res.*, 281:155–160.
119. Manoil, C., 1991, Analysis of membrane protein topology using alkaline phosphatase and β -galactosidase gene fusions. *Methods. Cell. Biol.*, 34:61–75.
120. Masoud, H., Altman, E., Richards, J.C., and Lam, J.S., 1994, General strategy for structural analysis of the oligosaccharide region of lipooligosaccharides. Structure of the oligosaccharide component of *Pseudomonas aeruginosa* IATS serotype 06 mutant R5 rough-type lipopolysaccharide. *Biochemistry*, 33:10568–10578.
121. Masoud, H., Sadvovskaya, I., de Kievit, T., Altman, E., Richards, J.C., and Lam, J.S., 1995, Structural elucidation of the lipopolysaccharide core region of the O-chain-deficient mutant strain A28 from *Pseudomonas aeruginosa* serotype 06 (International Antigenic Typing Scheme). *J. Bacteriol.*, 177:6718–6726.
122. Matewish, M.J., Nightingale, S.L., Levesque, R.C., and Lam, J.S., 1998, Molecular characterization of *Pseudomonas aeruginosa* *galE* and *rfpB* homologs and characterization of their roles in the biosynthesis of a novel lipopolysaccharide. Abstr. MSp9. In *Abstracts of the 48th Annual General Meeting of the Canadian Society of Microbiologists 1998*. Canadian Society of Microbiologists, Ottawa, ON.
123. Matewish, M.J., Walsh, A.G., and Lam, J.S., 1999, WapG, a galactosyltransferase essential for assembly of the lipopolysaccharide core of *Pseudomonas aeruginosa*. Abstr. 142. In *Abstracts of the American Society for Microbiology Conference on Pseudomonas '99: Biotechnology and Pathogenesis*. American Society for Microbiology, Washington, DC.
124. McClure, C.P., Rusche, K.M., Peariso, K., Jackman, J.E., Fierke, C.A., and Penner-Hahn, J.E., 2003, EXAFS studies of the zinc sites of UDP-(3-O-acyl)-N-acetylglucosamine deacetylase (LpxC). *J. Inorg. Biochem.*, 94:78–85.
125. Meier-Dieter, U., Starman, R., Barr, K., Mayer, H., and Rick, P.D., 1990, Biosynthesis of enterobacterial common antigen in *Escherichia coli*. Biochemical characterization of Tn10 insertion mutants defective in enterobacterial common antigen synthesis. *J. Biol. Chem.*, 265:13490–13497.
126. Meitert, T., 1964, Contribution a l'etude de la structure antigenique des *B. pyocyaneques*. II. Individualisation des groupes serologiques au moyen des antigenes 'O'. *Arch. Roum. Pathol. Microbiol.*, 23:679–692.
127. Mergaert, P., Van Montagu, M., Prome, J.C., and Holsters, M., 1993, Three unusual modifications, a D-arabinosyl, an N-methyl, and a carbamoyl group, are present on the Nod factors of *Azorhizobium caulinodans* strain ORS571. *Proc. Natl. Acad. Sci. USA*, 90:1551–1555.
128. Mohan, S. and Raetz, C.R., 1994, Endotoxin biosynthesis in *Pseudomonas aeruginosa*: Enzymatic incorporation of laurate before 3-deoxy-D-manno-octulosonate. *J. Bacteriol.*, 176:6944–6951.

129. Moreau, M., Richards, J.C., Fournier, J.M., Byrd, R.A., Karakawa, W.W., and Vann, W.F., 1990, Structure of the type 5 capsular polysaccharide of *Staphylococcus aureus*. *Carbohydr. Res.*, 201:285–297.
130. Morona, R., Mavris, M., Fallarino, A., and Manning, P.A., 1994, Characterization of the *rfc* region of *Shigella flexneri*. *J. Bacteriol.*, 176:733–747.
131. Morona, R., van den Bosch, L., and Manning, P.A., 1995, Molecular, genetic, and topological characterization of O-antigen chain length regulation in *Shigella flexneri*. *J. Bacteriol.*, 177:1059–1068.
132. Newton, G.J., Daniels, C., Burrows, L.L., Kropinski, A.M., Clarke, A.J., and Lam, J.S., 2001, Three-component-mediated serotype conversion in *Pseudomonas aeruginosa* by bacteriophage D3. *Mol. Microbiol.*, 39:1237–1247.
133. Nieto, J.M., Bailey, M.J., Hughes, C., and Koronakis, V., 1996, Suppression of transcription polarity in the *Escherichia coli* haemolysin operon by a short upstream element shared by polysaccharide and DNA transfer determinants. *Mol. Microbiol.*, 19:705–713.
134. Nikaido, H. and Hancock, R.E.W., 1986, Outer membrane permeability of *Pseudomonas aeruginosa*. In J. R. Sokatch (ed.), *The Bacteria, a Treatise on Structure and Function*, pp. 145–193. Academic Press, Orlando, FL.
135. Nikaido, H. and Vaara, M., 1985, Molecular basis of bacterial outer membrane permeability. *Microbiol. Rev.*, 49:1–32.
136. Olvera, C., Goldberg, J.B., Sánchez, R. and Soberón-Chávez, G., 1999, The *Pseudomonas aeruginosa* *algC* gene product participates in rhamnolipid biosynthesis. *FEMS Microbiol. Lett.*, 179:85–90.
137. Onishi, H.R., Pelak, B.A., Gerckens, L.S., Silver, L.L., Kahan, F.M., Chen, M.H., Patchett, A.A., Galloway, S.M., Hyland, S.A., Anderson, M.S., and Raetz, C.R., 1996, Antibacterial agents that inhibit lipid A biosynthesis. *Science*, 274:980–982.
138. Orgambide, G., Montrozier, H., Servin, P., Roussel, J., Trigalet-Demery, D., and Trigalet, A., 1991, High heterogeneity of the exopolysaccharides of *Pseudomonas solanacearum* strain GMI 1000 and the complete structure of the major polysaccharide. *J. Biol. Chem.*, 266:8312–8321.
139. Palleroni, N.J., 1993, *Pseudomonas* classification. A new case history in the taxonomy of gram-negative bacteria. *Antonie Van Leeuwenhoek*, 64:231–251.
140. Parolis, H., Parolis, L.A., and Olivieri, G., 1997, Structural studies on the *Shigella*-like *Escherichia coli* O121 O-specific polysaccharide. *Carbohydr. Res.*, 303:319–325.
141. Penketh, A., Pitt, T., Roberts, D., Hodson, M.E., and Batten, J.C., 1983, The relationship of phenotype changes in *Pseudomonas aeruginosa* to the clinical condition of patients with cystic fibrosis. *Am. Rev. Respir. Dis.*, 127:605–608.
142. Persson, B., Kallberg, Y., Oppermann, U., and Jönrvall, H., 2003, Coenzyme-based functional assignments of short-chain dehydrogenases/reductases (SDRs). *Chem. Biol. Interact.*, 143–144:271–278.
143. Pirrung, M.C., Tumey, L.N., McClerren, A.L., and Raetz, C.R., 2003, High-throughput catch-and-release synthesis of oxazoline hydroxamates. Structure-activity relationships in novel inhibitors of *Escherichia coli* LpxC: *In vitro* enzyme inhibition and antibacterial properties. *J. Am. Chem. Soc.*, 125:1575–1586.
144. Pirrung, M.C., Tumey, L.N., Raetz, C.R., Jackman, J.E., Snehaltha, K., McClerren, A.L., Fierke, C.A., Gantt, S.L., and Rusche, K.M., 2002, Inhibition of the antibacterial target UDP-(3-O-acyl)-N-acetylglucosamine deacetylase (LpxC): Isoxazoline zinc amidase inhibitors bearing diverse metal binding groups. *J. Med. Chem.*, 45:4359–4370.
145. Pitt, T.L., 1988, Epidemiological typing of *Pseudomonas aeruginosa*. *Eur. J. Clin. Microbiol. Infect. Dis.*, 7:238–247.

146. Poon, K.K.H., Mead, K., and Lam, J.S., 2003, Characterization WapR, a putative rhamnosyltransferase involved in core oligosaccharide biosynthesis from *Pseudomonas aeruginosa*. Abstr. D-242. In *Abstracts of the 103rd General Meeting of the American Society for Microbiology 2003*. American Society for Microbiology, Washington, DC.
147. Qureshi, S.T., Lariviere, L., Leveque, G., Clermont, S., Moore, K.J., Gros, P., and Malo, D., 1999, Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *J. Exp. Med.*, 189:615–625.
148. Radika, K. and Raetz, C.R., 1988, Purification and properties of lipid A disaccharide synthase of *Escherichia coli*. *J. Biol. Chem.*, 263:14859–14867.
149. Raetz, C.R. and Roderick, S.L., 1995, A left-handed parallel beta helix in the structure of UDP-*N*-acetylglucosamine acyltransferase. *Science*, 270:997–1000.
150. Raetz, C.R. and Whitfield, C., 2002, Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.*, 71:635–700.
151. Rahim, R., Burrows, L.L., Monteiro, M.A., Perry, M.B., and Lam, J.S., 2000, Involvement of the *rml* locus in core oligosaccharide and O polysaccharide assembly in *Pseudomonas aeruginosa*. *Microbiology*, 146:2803–2814.
152. Raymond, C.K., Sims, E.H., Kas, A., Spencer, D.H., Kutayin, T.V., Ivey, R.G., Zhou, Y., Kaul, R., Clendenning, J.B., and Olson, M.V., 2002, Genetic variation at the O-antigen biosynthetic locus in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 184:3614–3622.
153. Reddy, G.P., Hayat, U., Bush, C.A., and Morris, Jr., J.G., 1993, Capsular polysaccharide structure of a clinical isolate of *Vibrio vulnificus* strain BO62316 determined by heteronuclear NMR spectroscopy and high-performance anion-exchange chromatography. *Anal. Biochem.*, 214:106–115.
154. Reddy, G.P., Hayat, U., Xu, Q., Reddy, K.V., Wang, Y., Chiu, K.W., Morris, Jr., J.G., and Bush, C.A., 1998, Structure determination of the capsular polysaccharide from *Vibrio vulnificus* strain 6353. *Eur. J. Biochem.*, 255:279–288.
155. Reeves, P., 1993, Evolution of *Salmonella* O antigen variation by interspecific gene transfer on a large scale. *Trends Genet.*, 9:17–22.
156. Reeves, P.R., 1994, Biosynthesis and assembly of lipopolysaccharide. In A. Neuberger and L.L.M. van Deenen (ed.), *Bacterial Cell Wall, New Comprehensive Biochemistry*, pp. 281–314. Elsevier Science Publishers, New York.
157. Reeves, P.R., Hobbs, M., Valvano, M.A., Skurnik, M., Whitfield, C., Coplin, D., Kido, N., Klena, J., Maskell, D., Raetz, C.R., and Rick, P.D., 1996, Bacterial polysaccharide synthesis and gene nomenclature. *Trends Microbiol.*, 4:495–503.
158. Rick, P.D. and Osborn, M.J., 1977, Lipid A mutants of *Salmonella typhimurium*. Characterization of a conditional lethal mutant in 3-deoxy-D-mannooctulosonate-8-phosphate synthetase. *J. Biol. Chem.*, 252:4895–4903.
159. Rivera, M., Bryan, L.E., Hancock, R.E., and McGroarty, E.J., 1988, Heterogeneity of lipopolysaccharides from *Pseudomonas aeruginosa*: Analysis of lipopolysaccharide chain length. *J. Bacteriol.*, 170:512–521.
160. Rocchetta, H.L., Burrows, L.L., and Lam, J.S., 1999, Genetics of O-antigen biosynthesis in *Pseudomonas aeruginosa*. *Microbiol. Mol. Biol. Rev.*, 63:523–553.
161. Rocchetta, H.L., Burrows, L.L., Pacan, J.C., and Lam, J.S., 1998, Three rhamnosyltransferases responsible for assembly of the A-band D-rhamnan polysaccharide in *Pseudomonas aeruginosa*: A fourth transferase, WbpL, is required for the initiation of both A-band and B-band lipopolysaccharide synthesis. *Mol. Microbiol.*, 28:1103–1119.
162. Rowe, P.S. and Meadow, P.M., 1983, Structure of the core oligosaccharide from the lipopolysaccharide of *Pseudomonas aeruginosa* PAC1R and its defective mutants. *Eur. J. Biochem.*, 132:329–337.
163. Sadovskaya, I., Brisson, J.R., Lam, J.S., Richards, J.C., and Altman, E., 1998, Structural elucidation of the lipopolysaccharide core regions of the wild-type strain PAO1 and

- O-chain-deficient mutant strains AK1401 and AK1012 from *Pseudomonas aeruginosa* serotype O5. *Eur. J. Biochem.*, 255:673–684.
164. Sadvovskaya, I., Brisson, J.R., Thibault, P., Richards, J.C., Lam, J.S., and Altman, E., 2000, Structural characterization of the outer core and the O-chain linkage region of lipopolysaccharide from *Pseudomonas aeruginosa* serotype O5. *Eur. J. Biochem.*, 267:1640–1650.
165. Sánchez Carballo, P.M., Rietschel, E.T., Kosma, P., and Zähringer, U., 1999, Elucidation of the structure of an alanine-lacking core tetrasaccharide trisphosphate from the lipopolysaccharide of *Pseudomonas aeruginosa* mutant H4. *Eur. J. Biochem.*, 261:500–508.
166. Sandvik, O., 1960, Serological comparison between strains of *Pseudomonas aeruginosa* from human and animal sources. *Acta Pathol. Microbiol. Scand.*, 48:56–67.
167. Sau, S., Sun, J., and Lee, C.Y., 1997, Molecular characterization and transcriptional analysis of type 8 capsule genes in *Staphylococcus aureus*. *J. Bacteriol.*, 179:1614–1621.
168. Schnaitman, C.A. and Klena, J.D., 1993, Genetics of lipopolysaccharide biosynthesis in enteric bacteria. *Microbiol. Rev.*, 57:655–682.
169. Shashkov, A.S., Paramonov, N.A., Veremeychenko, S.P., Grosskurth, H., Zdorovenko, G.M., Knirel, Y.A., and Kochetkov, N.K., 1998, Somatic antigens of pseudomonads: Structure of the O-specific polysaccharide of *Pseudomonas fluorescens* biovar B, strain IMV 247. *Carbohydr. Res.*, 306:297–303.
170. Sompolinsky, D., Samra, Z., Karakawa, W.W., Vann, W.F., Schneerson, R., and Malik, Z., 1985, Encapsulation and capsular types in isolates of *Staphylococcus aureus* from different sources and relationship to phage types. *J. Clin. Microbiol.*, 22:828–834.
171. Sonnhammer, E.L., Eddy, S.R., Birney, E., Bateman, A., and Durbin, R., 1998, Pfam: Multiple sequence alignments and HMM-profiles of protein domains. *Nucleic Acids Res.*, 26:320–322.
172. Sorensen, P.G., Lutkenhaus, J., Young, K., Eveland, S.S., Anderson, M.S., and Raetz, C.R., 1996, Regulation of UDP-3-O-[R-3-hydroxymyristoyl]-N-acetylglucosamine deacetylase in *Escherichia coli*. The second enzymatic step of lipid A biosynthesis. *J. Biol. Chem.*, 271:25898–25905.
173. Stanislavsky, E.S. and Lam, J.S., 1997, *Pseudomonas aeruginosa* antigens as potential vaccines. *FEMS Microbiol. Rev.*, 21:243–277.
174. Stanislavsky, E.S., Lanyi, B., Knirel, Y.A., and Dmitriev, B.A., 1988, Chemotypes of *Pseudomonas aeruginosa*. *Zh. Microbiol. Epidemiol. Immunobiol.*, 5:1–14.
175. Steeghs, L., Berns, M., ten Hove, J., de Jong, A., Roholl, P., van Alphen, L., Tommassen, J., and van der Ley, P., 2002, Expression of foreign LpxA acyltransferases in *Neisseria meningitidis* results in modified lipid A with reduced toxicity and retained adjuvant activity. *Cell. Microbiol.*, 4:599–611.
176. Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrenner, P., Hickey, M.J., Brinkman, F.S., Huftnagle, W.O., Kowalik, D.J., Lagrou, M., Garber, R.L., Goltry, L., Tolentino, E., Westbrook-Wadman, S., Yuan, Y., Brody, L.L., Coulter, S.N., Folger, K.R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G.K., Wu, Z., Paulsen, I.T., Reizer, J., Saier, M.H., Hancock, R.E., Lory, S., and Olson, M.V., 2000, Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature*, 406:959–964.
177. Tassios, P.T., Gennimata, V., Maniatis, A.N., Fock, C., and Legakis, N.J., 1998, Emergence of multidrug resistance in ubiquitous and dominant *Pseudomonas aeruginosa* serogroup O11: The Greek *Pseudomonas Aeruginosa* Study Group. *J. Clin. Microbiol.*, 36:897–901.
178. Tobias, P.S., Soldau, K., and Ulevitch, R.J., 1986, Isolation of a lipopolysaccharide-binding acute phase reactant from rabbit serum. *J. Exp. Med.*, 164:777–793.
179. Trent, M.S., Ribeiro, A.A., Lin, S., Cotter, R.J., and Raetz, C.R., 2001, An inner membrane enzyme in *Salmonella* and *Escherichia coli* that transfers 4-amino-4-deoxy-L-arabinose to lipid A: Induction on polymyxin-resistant mutants and role of a novel lipid-linked donor. *J. Biol. Chem.*, 276:43122–43131.

180. Vachee, A., Scheftel, J.M., Husson, M.O., Izard, D., Ross, P., and Monteil, H., 1997, Tricentric study of the sensitivity of *Pseudomonas aeruginosa* serotyping to beta-lactams and aminoglycosides. *Pathol. Biol.* (Paris), 45:357–362.
181. Valdivia, R.H., Cirillo, D.M., Lee, A.K., Bouley, D.M., and Falkow, S., 2000, *mig-14* is a horizontally acquired, host-induced gene required for *Salmonella enterica* lethal infection in the murine model of typhoid fever. *Infect. Immun.*, 68:7126–7131.
182. Valdivia, R.H. and Falkow, S., 1997, Fluorescence-based isolation of bacterial genes expressed within host cells. *Science*, 277:2007–2011.
183. Valvano, M.A., Messner, P., and Kosma, P., 2002, Novel pathways for biosynthesis of nucleotide-activated *glycero-manno*-heptose precursors of bacterial glycoproteins and cell surface polysaccharides. *Microbiology*, 148:1979–1989.
184. Verder, E. and Evans, J., 1961, A proposed antigen schema for the identification of strains of *Pseudomonas aeruginosa*. *J. Infect. Dis.*, 109:183–193.
185. Virlogeux, I., Waxin, H., Ecobichon, C., and Popoff, M.Y., 1995, Role of the *viaB* locus in synthesis, transport and expression of *Salmonella typhi* Vi antigen. *Microbiology*, 141:3039–3047.
186. Walsh, A.G., Burrows, L.L., and Lam, J.S., 1999, Genetic and biochemical characterization of an operon involved in the biosynthesis of 3-deoxy-D-manno-octulosonic acid in *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.*, 173:27–33.
187. Walsh, A.G., Mawish, M.J., Burrows, L.L., Monteiro, M.A., Perry, M.B., and Lam, J.S., 2000, Lipopolysaccharide core phosphates are required for viability and intrinsic drug resistance in *Pseudomonas aeruginosa*. *Mol. Microbiol.*, 35:718–727.
188. Wang, J., Lory, S., Ramphal, R., and Jin, S., 1996, Isolation and characterization of *Pseudomonas aeruginosa* genes inducible by respiratory mucus derived from cystic fibrosis patients. *Mol. Microbiol.*, 22:1005–1012.
189. Wang, L. and Reeves, P.R., 1998, Organization of *Escherichia coli* O157 O antigen gene cluster and identification of its specific genes. *Infect. Immun.*, 66:3545–3551.
190. Waxin, H., Virlogeux, I., Kolyva, S., and Popoff, M.Y., 1993, Identification of six open reading frames in the *Salmonella enterica* subsp. *enterica* ser. Typhi *viaB* locus involved in Vi antigen production. *Res. Microbiol.*, 144:363–371.
191. Whitfield, C., 1995, Biosynthesis of lipopolysaccharide O antigens. *Trends Microbiol.*, 3:178–185.
192. Whitfield, C., Amor, P.A., and Koplin, R., 1997, Modulation of the surface architecture of gram-negative bacteria by the action of surface polymer:lipid A-core ligase and by determinants of polymer chain length. *Mol. Microbiol.*, 23:629–638.
193. Wilkinson, S.G., 1981, ³¹P N.m.r. evidence for the presence of triphosphate residues in lipopolysaccharides from *Pseudomonas aeruginosa*. *Biochem. J.*, 199:833–835.
194. Wilkinson, S.G., 1983, Composition and structure of lipopolysaccharides from *Pseudomonas aeruginosa*. *Rev. Infect. Dis.*, 5 (Suppl 5):S941–S949.
195. Williamson, J.M., Anderson, M.S., and Raetz, C.R., 1991, Acyl-acyl carrier protein specificity of UDP-GlcNAc acyltransferases from gram-negative bacteria: Relationship to lipid A structure. *J. Bacteriol.*, 173:3591–3596.
196. Wilson, D.B. and Hogness, D.S., 1969, The enzymes of the galactose operon in *Escherichia coli*. II. The subunits of uridine diphosphogalactose 4-epimerase. *J. Biol. Chem.*, 244:2132–2136.
197. Wokatsch, R., 1964, Serologische Untersuchungen an *Pseudomonas aeruginosa* (*Bact. Pyocyaneum*) aus verschiedenen Tierarten. *Zbl. Bakteriol. Abt. I. Orig.*, 192:468–476.
198. Wright, S.D., Ramos, R.A., Tobias, P.S., Ulevitch, R.J., and Mathison, J.C., 1990, CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science*, 249:1431–1433.

199. Wyckoff, T.J., Lin, S., Cotter, R.J., Dotson, G.D., and Raetz, C.R., 1998, Hydrocarbon rulers in UDP-*N*-acetylglucosamine acyltransferases. *J. Biol. Chem.*, 273:32369–32372.
200. Yang, H., Matewish, M., Loubens, I., Storey, D.G., Lam, J.S., and Jin, S., 2000, *migA*, a quorum-responsive gene of *Pseudomonas aeruginosa*, is highly expressed in the cystic fibrosis lung environment and modifies low-molecular-mass lipopolysaccharide. *Microbiology*, 146:2509–2519.
201. Ye, R.W., Zielinski, N.A., and Chakrabarty, A.M., 1994, Purification and characterization of phosphomannomutase/phosphoglucosyltransferase from *Pseudomonas aeruginosa* involved in biosynthesis of both alginate and lipopolysaccharide. *J. Bacteriol.*, 176:4851–4857.
202. Yethon, J.A., Vinogradov, E., Perry, M.B., and Whitfield, C., 2000, Mutation of the lipopolysaccharide core glycosyltransferase encoded by *waaG* destabilizes the outer membrane of *Escherichia coli* by interfering with core phosphorylation. *J. Bacteriol.*, 182:5620–5623.
203. Yethon, J.A. and Whitfield, C., 2001, Purification and characterization of WaaP from *Escherichia coli*, a lipopolysaccharide kinase essential for outer membrane stability. *J. Biol. Chem.*, 276:5498–5504.
204. Young, K., Silver, L.L., Bramhill, D., Cameron, P., Eveland, S.S., Raetz, C.R., Hyland, S.A., and Anderson, M.S., 1995, The *envA* permeability/cell division gene of *Escherichia coli* encodes the second enzyme of lipid A biosynthesis. UDP-3-*O*-(*R*-3-hydroxymyristoyl)-*N*-acetylglucosamine deacetylase. *J. Biol. Chem.*, 270:30384–30391.
205. Zhao, X., Creuzenet, C., Bélanger, M., Egbosimba, E., Li, J., and Lam, J.S., 2000, WbpO, a UDP-*N*-acetyl- α -galactosamine dehydrogenase from *Pseudomonas aeruginosa* serotype O6. *J. Biol. Chem.*, 275:33252–33259.
206. Zhao, X. and Lam, J.S., 2002, WaaP of *Pseudomonas aeruginosa* is a novel eukaryotic type protein-tyrosine kinase as well as a sugar kinase essential for the biosynthesis of core lipopolysaccharide. *J. Biol. Chem.*, 277:4722–4730.
207. Zhao, X., Wenzel, C.Q., and Lam, J.S., 2002, Nonradiolabeling assay for WaaP, an essential sugar kinase involved in biosynthesis of core lipopolysaccharide of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.*, 46:2035–2037.
208. Zhou, Z., White, K.A., Polissi, A., Georgopoulos, C., and Raetz, C.R., 1998, Function of *Escherichia coli* MsbA, an essential ABC family transporter, in lipid A and phospholipid biosynthesis. *J. Biol. Chem.*, 273:12466–12475.
209. Zielinski, N.A., Chakrabarty, A.M., and Berry, A., 1991, Characterization and regulation of the *Pseudomonas aeruginosa* *algC* gene encoding phosphomannomutase. *J. Biol. Chem.*, 266:9754–9763.

ALGINATE BIOSYNTHESIS

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1. INTRODUCTION

Alginate was first isolated from marine algae in the 19th century, and was first identified from a bacterial source, namely mucoid *Pseudomonas aeruginosa*, in the 1960s⁷⁵. Alginate is also synthesized by *Azotobacter vinelandii* as part of the encystment process⁵⁴. Alginate is a simple unbranched polysaccharide that is composed of two kinds of uronic acid residues: β -D-mannuronic acid (M), and its C5 epimer, α -L-guluronic acid (G) (Figure 1A). Excellent reviews on alginate research have been offered by the late Peter Gacesa in 1990⁴⁶ and 1998⁴⁵. The pathogenesis of mucoid, alginate-producing *P. aeruginosa* in cystic fibrosis (CF) patients was also reviewed in 1996 by Govan and Deretic⁵⁶. The following chapter presents highlights from these previous reviews and new studies that have recently been described.

The sugar subunits are linked by β -1,4 glycosidic linkages. These monomers may be arranged in polymannuronic (–M–M–M–M–) blocks, polyguluronate blocks (–G–G–G–G–), or in heteropolymeric blocks (–M–G–M–G–M–G–). In the case of *P. aeruginosa*, alginate is present in either M blocks or M–G blocks, but never G blocks, as demonstrated by ¹H-nuclear magnetic resonance (NMR) analyses^{14, 126}. From the Haworth projection of monosaccharide subunits (Figure 1B), it is evident that the physical structure of the polymer depends on the block structure. Polymannuronate regions have di-equatorial linkages that result in the formation of flexible

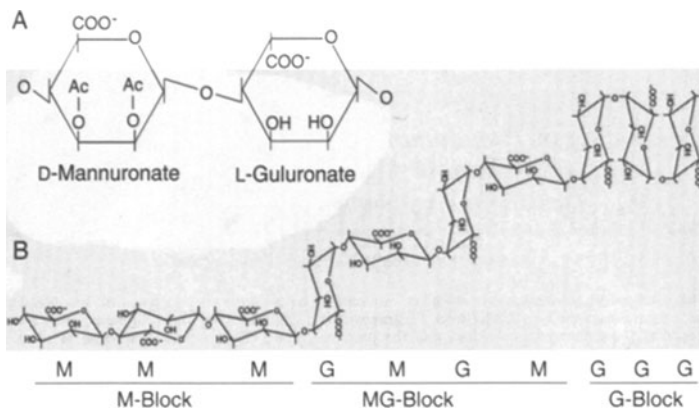


Figure 1. A. Monomers of alginate, D-mannuronate (M) and its C-5 epimer, L-guluronate (G), linked with a β -1,4 glycosidic bond. O acetyl groups (Ac) are found at carbons 2 and/or 3 of only M residues. B. Monomers in alginate are arranged in block structures, here shown in their favored chair conformations: M blocks, M-G blocks, and G-blocks. However, *Pseudomonas* alginates are devoid of G-blocks. Glycosidic bonds between M residues result in ribbon-like structures, and G residues introduce a bend in the linear chain, which affects the physical properties of the polymer¹⁴.

ribbon-like gels. Polyguluronate regions have di-axial linkages that result in the formation of a rigid buckled chain⁴⁷ (Figure 1). Hence, alginates rich in polyG make firm but brittle gels, whereas those rich in polyM or polyMG stretches are more elastic. Additionally, alginates that contain polyG blocks bind strongly to calcium ions, and this contributes to the formation of rigid gels¹³². *A. vinelandii* makes alginates with G blocks in the process of encystment⁶³. In bacterial alginates, some of the mannuronic acid residues are O acetylated either in the C2 or C3 or both positions¹³⁰. The high molecular weight and negative charge ensures that the polysaccharide is very hydrated and viscous. Extensive O acetylation of alginate increases its water binding capacity, which in turn determines the extent of viscosity¹³². The degree of acetylation also plays a role in determining its sensitivity to the alginate degrading enzymes, called alginate lyases, with acetylated alginate being less susceptible to lyases than nonacetylated alginate¹³¹.

2. *P. AERUGINOSA* AND CHRONIC INFECTION OF CF PATIENTS

P. aeruginosa is an opportunistic pathogen that can cause severe and life-threatening infections in immunosuppressed hosts, such as patients with respiratory diseases, burns, cancers undergoing chemotherapy, and with CF. Virulence factors produced by *P. aeruginosa* include numerous extracellular

toxins, proteases, hemolysins, and exopolysaccharides that play a role depending upon the type of infection. The striking feature of *P. aeruginosa* strains infecting the CF pulmonary tract is their mucoid conversion *in vivo*, a phenotype that is indicative of alginate overproduction^{35, 75}. This is often referred to here as the Alg⁺ phenotype. Alginate is a capsule-like exopolysaccharide that loosely adheres to the cells, and so most of it is found in the culture's supernatant. Alg⁺ correlates with the ability of *P. aeruginosa* to persist in the lungs of CF patients and cause chronic bronchopulmonary infection⁵⁷.

The initial and intermittent colonization of the CF lungs by *P. aeruginosa* can be eradicated by early aggressive antibiotic therapy⁴³. However, when the colony morphology of bacteria, isolated from sputum samples, is observed to convert to the mucoid form, the organisms can no longer be eliminated from the lungs despite aggressive antibiotic therapy⁴³. The appearance of mucoid strains correlates with the formation of a bacterial biofilm containing microcolonies, the development of anti-*P. aeruginosa* antibodies, inflammation, and a generally poor prognosis for the patient^{62, 73, 101}. Despite a seemingly intact host immune response¹⁰⁵, the CF patient is eventually overwhelmed by the chronic infection as the number of *P. aeruginosa* in the sputum becomes as high as 10⁸ cfu/ml¹²⁴.

There have been dramatic advances in our knowledge of the molecular and cellular basis of CF¹²⁵. This common autosomal recessive disease is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a phosphorylation-regulated Cl⁻ channel in the apical membrane of involved epithelia. CF disease is characterized by chronic airway infection that progressively destroys the lung and often leads to respiratory failure. By far the most common pathogen responsible for the morbidity and mortality seen in these patients is *P. aeruginosa*⁷². There is clearly an association between the CF defective transepithelial Cl⁻ transporter and the propensity for chronic airway infection by *P. aeruginosa* in these patients. There is evidence that CFTR contributes to host defenses by acting as a bacterial ligand, and thus the CF defect may prevent normal clearance of *P. aeruginosa* from the respiratory tract¹⁰⁷. Also, the bactericidal activity against *P. aeruginosa* that is normally present in airway surface fluids of epithelia is reported to have reduced effectiveness in the CF lung environment¹³³.

3. ROLES OF ALGINATE IN *P. AERUGINOSA* PATHOGENESIS

About 80% of the *P. aeruginosa* isolates from the CF patient undergo mucoid conversion, whereas only about 1% of clinical *P. aeruginosa* isolates from other types of infections are Alg⁺ (see refs [32], [33], [140]). Thus, alginate production appears to have an important role in the unique host-parasite

relationship between the CF patient and *P. aeruginosa*. There is evidence that the Alg⁺ phenotype confers several selective advantages to the bacterial invader, which has been thoroughly reviewed⁵⁶ and includes the following:

- Alginate confers increased resistance to phagocytosis, a property typically associated with bacterial capsules. Mucoid organisms show a reduced susceptibility to antibody-dependent bactericidal mechanisms^{1, 123}. Alginate antibodies that form are usually nonopsonic¹⁰⁹. Alginate also affects leukocyte functions, such as the oxidative burst and interference with opsonization, and plays an immunomodulatory role via induction of proinflammatory lymphokines and suppression of lymphocyte transformation^{101, 102}. Alginate has been shown to neutralize oxygen radicals, such as those released by activated polymorphonuclear leukocytes (PMNs)¹²⁹. Mucoid organisms are also hyperinduced for the production of the manganese form of superoxide dismutase, which can detoxify oxygen radicals^{59, 60}.
- Alginate appears to be part of an adherence mechanism. Autopsies show that mucoid *P. aeruginosa* forms adherent microcolonies in the lung⁷³. The role of alginate as an adhesin has also been demonstrated in vitro^{82, 113}. The biofilm formed by mucoid organisms contains microcolonies, which is unique. Although alginate is not required for biofilm formation, a mutant defective in alginate biosynthesis (*algD::Tn501*) clearly shows a lag in initial biofilm development, suggesting a role in early attachment⁹⁶.
- Alginate released from the organisms is highly viscous in aqueous solution, and probably contributes to the extremely viscous environment containing abnormal bronchial secretions in the CF lung and nucleic acids from dead phagocytes. This results in obstruction of small airways, interference with mucociliary airway clearance, and impaired movement of phagocytes.
- Alginate provides a polyanionic barrier that may exclude cationic peptide antibiotics¹⁰¹. Alginate lyase treatment of mucoid *P. aeruginosa* can improve the efficacy of administered antibiotics⁴.
- The Alg⁺ phenotype also correlates with variation in the expression of other virulence mechanisms, including a decrease in the production of proteases^{93, 98} and other specific virulence determinants, such as exotoxin A, exoenzyme S, phospholipase C, and pyochelin levels^{78, 147}. Lipopolysaccharide (LPS) is a major virulence factor for this species¹⁸, but it is also a major target antigen for protective antibodies¹⁹. CF strains frequently convert to LPS-rough as a result of mutations affecting production of O side chains^{58, 78}. This down-regulation in the production of virulence factors associated with mucoid

conversion is consistent with a tendency in chronic host–parasite relationships to progress toward a state of commensalism¹⁴⁷ and suggests that alginate regulation has multisystem involvement.

4. MECHANISM OF MUCOID CONVERSION

The alternative sigma factor σ^{22} encoded by *algT* (also called *algU*) is essential for alginate production^{37, 38}. AlgT shows high sequence similarity (66% identity) to sigma E (σ^E) of *Escherichia coli*^{29, 31, 77}. σ^{22} (AlgT/AlgU) can function as a sigma factor in in vitro transcription assays⁶¹. From a plasmid clone (6–8 copies/cell), *algT* activates the Alg⁺ phenotype in otherwise nonmucoid strains of *P. aeruginosa* and several other *Pseudomonas* species⁵⁰. The *algT* product positively regulates its own transcription³¹. The *algT* gene is in an operon (Figure 2A) with four other genes called *mucA-mucB-mucC-mucD* (previously referred to as *mucA-algN-algM-algY*, respectively⁹⁹).

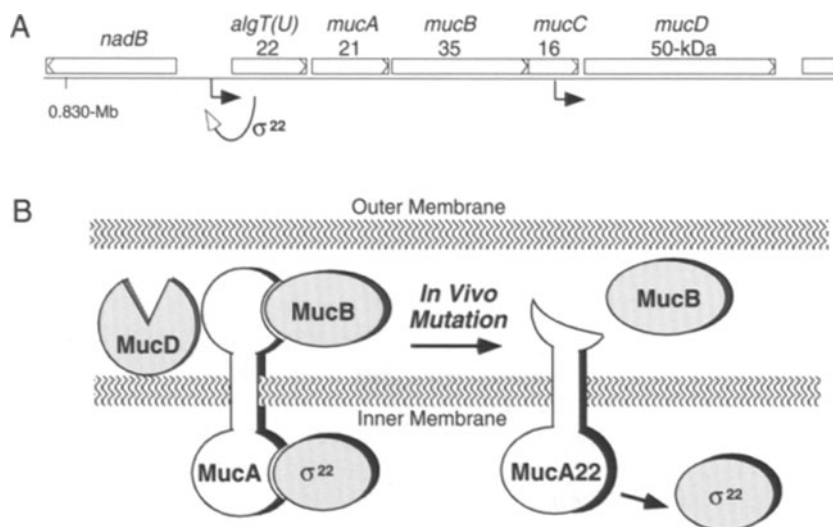


Figure 2. A. Map of the *algT(algU)* operon of *P. aeruginosa*. Numbers under genes show the molecular weights (kDa) of each gene product. Closed arrowheads indicate promoter regions, and the open arrowhead indicates autoregulation by the *algT* product, σ^{22} . Adjacent *nadB* maps at 0.830-Mb on the chromosome. B. Model for control of σ^{22} activity via sequestration by the transmembrane protein, MucA. MucB appears to interact with the periplasmic domain of MucA as part of signal transduction mechanism. Periplasmic MucD may play a role in signal transduction. *P. aeruginosa* strains in CF patients frequently undergo *mucA22* mutation, which truncates MucA, resulting in deregulated σ^{22} molecules that are free to transcribe its specific promoters.

Mutations in *mucA* are typically responsible for the mucoid conversion observed in clinical isolates⁸⁴. A high overall frequency of *mucA* alterations in mucoid CF isolates has been reported⁹. A common mutation, called the *mucA22* allele, has a single bp deletion in a string of 5 Gs that results in a prematurely truncated MucA. The CF strain FRD1 (commonly used in this laboratory) has the *mucA22* allele and is highly mucoid³¹.

MucA is an inner membrane protein with one transmembrane domain (Figure 2B), and appears to be the main regulator to post-translationally control σ^{22} activity⁸⁷. MucA acts as an anti-sigma factor that binds and sequesters σ^{22} , thus affecting its ability to transcribe in vitro¹²². MucB(AlgN) is also a negative regulator⁵⁰. MucB is a periplasmic protein that probably binds the periplasmic domain of MucA^{87, 122}. Mutation in *mucB* leads to a slightly mucoid phenotype, probably due to the lack of MucA–MucB interactions⁸³. Thus, MucA–MucB apparently forms a signal transduction complex that normally keeps σ^{22} transcriptional activity low, except in the event of unknown environmental signals. However, CF strains often bypass this circuit with a *mucA* defect (e.g., *mucA22*, truncating the periplasmic domain; see Figure 2B), which deregulates control of σ^{22} transcriptional activity at promoters with the consensus sequence GAACTT (–35)–TCtga (–10)^{31, 56}. The function of MucC, which is translationally coupled to MucB, is generally unknown but has been reported as both a positive and negative regulator^{8, 99}. MucD has high similarity to *E. coli* HtrA (DegP), a periplasmic serine protease involved in the proteolysis of abnormal proteins and is required for resistance to oxidative and heat stress^{9, 99}. The *mucD* gene also has a secondary promoter, independent of σ^{22} , within the *algT* operon¹⁴⁶.

The selective pressure for mucoid conversion in CF isolates of *P. aeruginosa* has received much speculation. The environment of the CF lung, particularly high osmolarity and dehydration, has been reported to contribute to the activation of the *algD* promoter^{7, 30}. The appearance of mucoid strains in CF patients correlates with the formation of a bacterial biofilm containing microcolonies and inflammation^{62, 73, 102}. The inflammatory defense mechanism against mucoid *P. aeruginosa* is dominated by PMNs and antibodies. *P. aeruginosa* in biofilms, as seen in the CF lung, has been shown to activate the oxidative burst of PMNs^{1, 68}. During phagocytosis of the bacteria, oxygen free radicals are produced, which generate oxidative stress and lead to further inflammation^{12, 64}. Activated PMNs, and/or their release of toxic oxygen by-products in the CF lung environment may play a role in the generation and/or selection of mucoid variants during the inflammatory response to *P. aeruginosa*. When *P. aeruginosa* PAO1 is grown as a biofilm in flow-cells and repeatedly exposed to activated human peripheral PMNs, the bacteria in the effluent of the flow cell contain Alg⁺ cells⁸⁶. This suggests that mucoid conversion of *P. aeruginosa* in the CF lung may result from interaction with PMNs. When

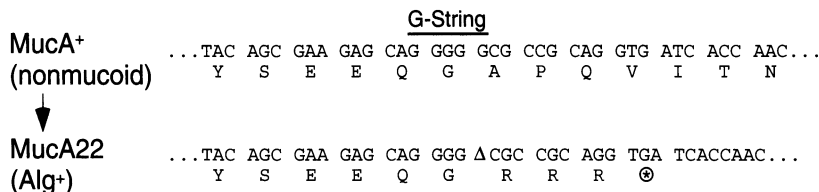


Figure 3. Sequence of the region of *mucA* associated with the common *mucA22* allele. Alg⁺ variants of PAO1 in a biofilm after being subjected to H₂O₂ treatment showed deletion of a G residue in a string of 5 G residues located at 426–430 bp in the *mucA* ORF, resulting in the *mucA22* allele and premature termination of transcription as indicated by the asterisk⁸⁶.

PAO1 biofilms are treated with low levels of hydrogen peroxide, as if released by the PMNs, the formation of mucoid variants is also detected⁸⁶. The *mucA* regions of these Alg⁺ strains show the typical *mucA22* allele, with a deletion of a G residue in the string of five guanosines located at 426–430 bp in the *mucA* open reading frame (Figure 3). Thus, PMNs and their oxygen reactive products can cause the phenotypic and genotypic changes, which are so typical of the intractable form of *P. aeruginosa* in the CF lung⁸⁶.

Another interesting property associated with mucoid *P. aeruginosa* strains from CF patients is that the Alg⁺ phenotype is typically unstable in the laboratory, especially under nonaerated conditions⁹⁷. The genetic alterations responsible for spontaneous nonmucoid conversion were closely linked to *algT*, and the possibility of a genetic ‘switch’ called *algS* was originally speculated³⁸. However, spontaneous nonmucoid variants are usually due to mutations in the *algT* gene itself^{31, 121}. These *algT* mutations appear to be extragenic suppressors of the *mucA* mutations, which provide genetic evidence for a σ^{22} –MucA interaction. It is curious that Alg⁺ to nonmucoid reversion does not occur by a mutation that simply restores the wild-type *mucA* allele³¹. Static growth of mucoid *P. aeruginosa* also selects for nonmucoid variants that have acquired flagellum-dependent motility¹⁵².

5. THE ALGINATE BIOSYNTHESIS OPERON

Mutations in alginate biosynthetic genes were observed to cluster adjacent to *argF*²². This was shown to constitute a large operon, with no required internal promoters, based on the fact that Tn501 insertions in the gene cluster are polar on all downstream genes¹⁵. The *algD* gene is proximal to the promoter of the operon, which is generally referred to here as the *algD* operon. The same cluster of 12 genes (Figure 4) has been found in other bacteria that synthesize alginate, including *Pseudomonas fluorescens*⁹⁵, *Pseudomonas syringae*¹⁰³, and *Azotobacter vinelandii*¹¹⁵. However, in *A. vinelandii* there



Figure 4. Map of the *algD* operon for alginate biosynthesis in *P. aeruginosa*. Closed arrowhead indicates the promoter region, which is under σ^{22} control. Open arrowhead indicates control by transcriptional regulators (AlgR, AlgZ, AlgB). Adjacent *argF* maps at 3.960-Mb on the PAO1 chromosome map.

appears to be multiple promoters in the alginate cluster^{76, 142}. The promoter of the *algD* operon in *P. aeruginosa* shows consensus with the σ^{22} promoters, and σ^{22} activates its transcription in in vitro transcription assays⁶¹. The roles of the *algD* operon gene products in alginate biosynthesis are described below.

6. HIERACHICAL REGULATION OF ALGINATE PRODUCTION

The *algD* operon of 12 genes is located at 3.96-Mbp on the 6.26-Mb physical genomic map of strain PAO1¹³⁵. The *algD* operon is nearly silent in typical *P. aeruginosa* strains, but shows remarkably high activity in mucoid strains²⁷. The regulation of the *algD* operon is complex and consists of a variety of factors encoded by genes around the chromosome (Figure 5). In addition to σ^{22} , encoded by *algT/algU* mapping at 0.83-Mb, there are 3 major regulators known for *algD* expression, called AlgR, AlgZ, and AlgB:

- The *algR* (*algR1*) gene, located at 5.92-Mb on the PAO1 chromosome map, encodes a two-component regulator. AlgR (also called AlgR1) contains a conserved N-terminal domain typical of response regulators, but has an undefined C-terminal domain²⁶. The sensory component interacting with AlgR is FimS (also called AlgZ) and is encoded by the upstream gene¹⁴³. FimS does not possess sequence similarity to typical histidine protein kinases^{143, 153}. The AlgR-FimS pair are both required for type-4 pilin mediated motility (i.e., twitching motility) on a solid surface, however, only AlgR is required for alginate production^{79, 143, 144}. AlgR footprints on two segments of the *algD* promoter⁶⁹ and three regions of the *algC* promoter⁴⁴. The *fimS*-*algR* operon is partially under σ^{22} control^{143, 151}.
- The *algZ* gene (mapping at 3.79-Mbp) encodes AlgZ (not to be confused with FimS/AlgZ above) which binds a sequence located 280 bp

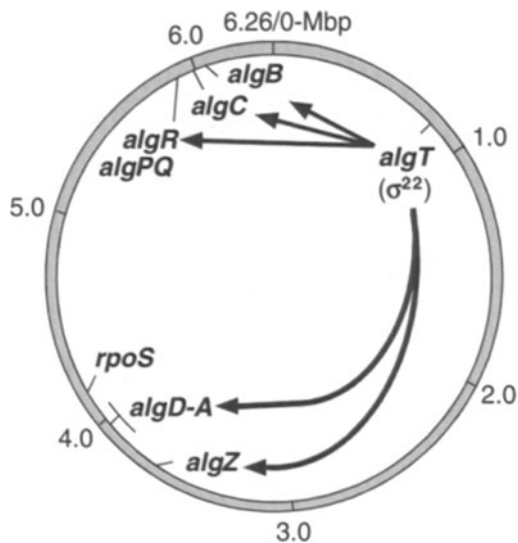


Figure 5. Circular chromosome map in Mbp of the PAO1 chromosome¹³⁵ showing the location of genes associated with alginate production (see www.pseudomonas.com for more details). The *algD-A* operon and *algC* encode enzymes for alginate biosynthesis. The other genes encode products that control (directly or indirectly) the *algD* promoter. Arrows point to genes under σ^{22} control in a hierarchy of gene regulation¹⁵¹.

upstream of the *algD* promoter⁵. Expression of *algZ* is dependent on AlgT but not AlgB or AlgR. Mutagenesis of AlgZ's 36-bp footprint in an *algD-cat* fusion blocked *algD* expression, indicating that AlgZ acts as an activator of *algD* transcription⁵. AlgZ is only 12 kDa in size and resembles the Arc repressors of the ribbon–helix–helix family of DNA binding proteins. An *algZ* mutant of Alg⁺ strain FRD1 shows no alginate production or detectable *algD* transcription⁶, indicating that this is an important regulator of alginate biosynthesis.

- The *algB* gene maps at 6.17-Mb on the chromosome and encodes a classic two-component regulator. The *algB* gene was among the first alginate genes to be cloned, and is a positive regulator of *algD* expression at 37°C^{52, 53}. The mechanism of AlgB regulation of alginate production is still unclear and probably does not involve direct binding at the *algD* promoter^{148, 150}. AlgB is a response regulator of the NtrC class of transcription enhancer elements that typically utilize σ^{54} (σ^N) promoters¹⁵⁰. σ^{22} controls the level of *algB* expression¹⁴⁹. Downstream of *algB* is *kinB*, which encodes a typical histidine kinase that is apparently the environmental sensor of AlgB⁸⁰. KinB is autophosphorylated at His-385 and transfers the phosphate to Asp-59 of AlgB⁸⁰ (Figure 6). However, KinB is not

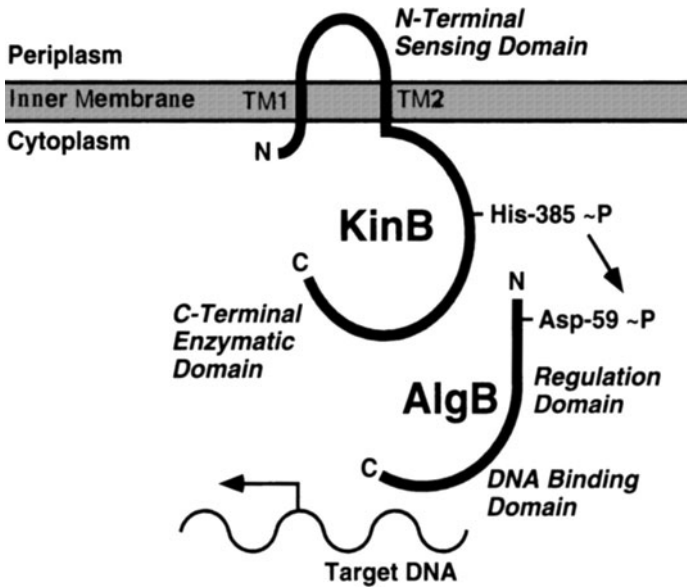


Figure 6. Model of the AlgB–KinB two-component regulatory complex. Membrane-spanning KinB has a periplasmic N-terminal sensing domain and a cytoplasmic C-terminal autophosphorylation domain that transfers the phosphate to the N-terminal regulation domain of AlgB⁸⁰. However, neither AlgB phosphorylation nor KinB is required for the Alg⁺ phenotype⁷⁹.

required for the Alg⁺ phenotype, suggesting that phosphorylated AlgB has a distinct and different regulatory function⁷⁹. The *algB*–*kinB* pair forms an operon, and so both are under σ^{22} control.

Other regulators of *algD* expression have also been identified. AlgQ (Algr2) is a positive regulator of nucleoside diphosphokinase (NDK). This is necessary for the formation of GDP-mannose, which is required for alginate biosynthesis (see below) and potentially other virulence properties⁷¹. AlgP (Algr3) is a highly basic histone-like protein required for normal *algD* expression^{28, 70}. Integration host factor may play a role in *algD* expression¹⁴⁹. RpoS, the stationary-phase sigma factor, is also required for high-level alginate production¹³⁶.

7. ECF SIGMA FACTOR SYSTEMS SIMILAR TO σ^{22}

The *algT mucABCD* gene cluster controls alginate production but also appears to encode a larger stress response apparatus. The operon bears

striking similarity to stress response systems in other bacteria. σ^{22} -related sigma factors are members of the ECF (extra cytoplasmic function) family of transcription factors that respond to membrane stresses^{77, 92}. *A. vinelandii* has the same arrangement of genes as in *P. aeruginosa*, which are also involved in alginate production, but are associated with encystment in a stress response to unfavorable environments⁸⁵. The *rpoE* cluster in *E. coli* is similar to *P. aeruginosa*, except that *htrA/degP*, the *mucD* equivalent, is missing¹¹⁷. σ^E is an extreme heat shock sigma factor that was discovered through studies on heat shock survival mechanisms³⁴. σ^E is under the control of RseAB, which resemble MucAB, and regulates *degP* (encoding a MucD-like serine endoprotease required for heat resistance), *rpoH* (encoding heat shock sigma factor σ^{32} for transcription of chaperones DnaK/J, GroES/EL), as well as its own *rpoE* promoter^{25, 91}. A broad role for σ^E as an extracytoplasmic periplasmic stress response pathway is emerging through studies on the genetics and physiology of *E. coli*'s response to cell envelope protein folding defects¹¹². σ^E is reported to be an essential sigma factor at all temperatures, and that cells lacking σ^E are able to grow as a result of unlinked suppressor mutations²⁴. *E. coli* σ^E is sufficiently similar to *P. aeruginosa* σ^{22} that *rpoE* can complement an *algT(algU)* mutation in *P. aeruginosa*, restoring the Alg⁺ phenotype¹⁵⁴.

Evidence is emerging that *P. aeruginosa* σ^{22} controls several genes other than those for alginate production. A proteomic study, using a 2D-gel analysis of all cellular proteins, showed that unregulated σ^{22} in Alg⁺ PAO1-*mucA22* cells causes elevated transcription of *dsbA*, encoding disulfide bond isomerase⁸¹. A genomic analysis using a DNA microarray analysis shows that the expression of numerous genes are elevated in Alg⁺ PAO1³⁶. Thus, the *algT-mucABCD* encoded membrane complex is probably a signal transduction mechanism that responds to environmental stress.

8. SYNTHESIS OF A NUCLEOSIDE DIPHOSPHO-SUGAR PRECURSOR OF ALGINATE

A putative pathway for alginate biosynthesis was first described for the brown seaweed *Fucus gardneri* based on enzyme activities detected⁷⁴. Early studies on bacterial alginate suggested a similar pathway, in which the main precursor is the nucleoside diphospho(NDP)-sugar, GDP-mannuronate (Figure 7). This was based on detection of the necessary enzyme activities, albeit at low levels, in alginate-producing strains of *A. vinelandii*¹¹¹ and *P. aeruginosa*¹¹⁰. Studies with mutants of mucoid strain FRD1 defective in carbohydrate metabolism showed the flow of carbon into alginate and demonstrated

fructose-1,6-bisphosphate via the Entner–Doudoroff pathway enzymes and FDA³. The following four steps are believed to convert fructose-6-phosphate to the primary precursor, GDP-mannuronate.

Step 1 in the synthesis of GDP-mannuronate is the conversion of fructose-6-phosphate to mannose-6-phosphate by phosphomannose isomerase (PMI). Some controversy over the pathway developed because PMI activity in mucoid *P. aeruginosa* cells has been difficult or impossible to detect. Also, because a PGI defective mutant could not synthesize alginate directly from mannitol (see Figure 7), it suggested that fructose-6-phosphate is not a precursor of alginate². However, when the *pmi* (*manA*) gene of *E. coli* encoding PMI was found to complement a nonmucoid *algA43* mutant of *P. aeruginosa* 8830 back to the Alg⁺ phenotype, it strongly suggested that PMI activity is indeed central to the alginate pathway²¹. This *pmi* gene in *P. aeruginosa* has become known as *algA*. Interestingly, however, the inferred amino acid sequence of AlgA does not resemble PMI (ManA) of *E. coli*²⁰. However, when *algA* was overexpressed, its 56-kDa protein product catalyzed the unidirectional conversion of fructose-6-phosphate to mannose-6-phosphate, indicating PMI activity⁴⁸.

Step 2 is the production of mannose-1-phosphate by phosphomannose mutase (PMM), an activity found in AlgC⁸⁹. The *algC* gene is not in the *algD* operon but is distantly located at 5.99-Mbp on the chromosome. AlgC is also involved in the biosynthesis of LPS⁵¹, but is reported to be up-regulated with other alginate genes in mucoid strains⁴⁴. AlgC also has phosphoglucose mutase (PGM) activity, which is important in the synthesis of O-side chains of LPS¹⁷ and rhamnolipids¹⁰⁰.

Step 3 is the formation of GDP-mannose by GDP-mannose pyrophosphorylase (GMP). Analysis of AlgA revealed that it is a bifunctional enzyme with two separate activities that include GMP (step 3) as well as PMI activity (step 1). This was demonstrated by the co-elution of both activities through successive rounds of chromatographic separations¹²⁷. It is interesting that this bifunctional enzyme catalyzes two reaction steps that are not contiguous. When compared to the current database, the AlgA sequence shows high homology to GMP enzymes like ManC of *E. coli*.

Step 4 requires GDP-mannose dehydrogenase (GMD), with NAD⁺ as a cofactor, to form GDP-mannuronic acid, the primary NDP-sugar precursor of alginate. GDP-mannose is converted to the uronic acid form by undergoing oxidation of the primary hydroxyl group at C6 of mannose to the corresponding carboxylic acid. It is interesting that GMD activity is low in the extracts of Alg⁺ *P. aeruginosa*, but GMD activity is elevated when the 48-kDa product of the *algD* gene is overexpressed^{27, 118}. Metabolic studies indicate that GMD is a key regulatory enzyme and committal step in alginate biosynthesis^{138, 139}. The crystal structure of AlgD shows that it forms a dimer¹³⁴.

9. POLYMER FORMATION

In that epimerization of mannuronate residues to guluronate occurs at the polymer level in the periplasm (see below), the initial polymeric product of the alginate biosynthetic pathway is apparently polymannuronate (polyM). It is likely that polymerization and secretion across the inner membrane occurs simultaneously, and there is no evidence for an isoprenoid lipid carrier. The alginate polymerase has not yet been identified, but the most likely candidate is Alg8, which bears structural homology to β -glycosyl transferases. This analysis is based on a sequence comparison method called hydrophobic cluster analysis, which can show three-dimensional similarities in proteins with limited sequence relatedness¹¹⁹. Glycosyl transferases catalyze the transfer of sugar residues from an activated donor substrate, often a NDP-sugar, to an acceptor molecule, which in most cases is a growing carbohydrate chain. Glycosyl transferases fall predominantly into two classes: Nonprocessive enzymes that transfer a single sugar residue to the acceptor and processive enzymes that transfer dual sugar residues to the acceptor. There are also two major catalytic mechanisms, called retaining and inverting, that describe product stereochemistry and thus determine α or β linkage¹¹⁹. Among the 26 families of NDP-sugar glycosyltransferases, Alg8 best resembles members of Family 2, a large group of processive and inverting glycosyl transferases, suggesting that alginate grows two monomer units at a time¹³. This is also consistent with the β -1,4 linkages in alginate.

10. POLYMER-LEVEL EPIMERIZATION OF MANNURONATE TO GULURONATE

AlgG is a polymer-level D-mannuronate C5-epimerase in *P. aeruginosa*, which is responsible for converting D-mannuronates (M) to L-guluronate (G) in alginate by rotation at C5 (see Figure 1). AlgG was identified using a genetic approach in which the Alg⁺ strain FRD1, which produces alginate with a M/G ratio of about 60/40, was mutagenized with nitrosoguanidine and plated on an agar medium containing a G-specific alginate lyase from *Klebsiella*¹⁴. Wild-type colonies lose their mucoid appearance on G-lyase agar because alginate chains are cleaved at G residues, whereas C5-epimerase mutants retain the mucoid phenotype. Purified alginate from an *algG4* mutant was shown by ¹H-NMR spectroscopy to be polyM. A clone of the wild-type *algD* operon complemented the mutation, and polar Tn501 mutagenesis of the clone physically located *algG*¹⁴. The amino acid sequence of AlgG was unlike any known protein except for an inferred signal peptide. Pulse-chase labeling studies showed that the 60-kDa preAlgG is processed to a periplasmic 55-kDa

mature AlgG. Expression of *algG* from a plasmid in the *algG4* (i.e., polyM producing) mutant of *P. aeruginosa* restored production of an alginate containing L-guluronate residues, but it was not possible to exceed 50% G residues despite overexpression³⁹.

The localization of AlgG to the periplasm suggested that it may be a polymer-level mannuronan C5-epimerase. Indeed, when recombinant AlgG is incubated with polyM produced by the *algG4* mutant, epimerization of M to G residues is observed³⁹. However, the in vitro epimerization reaction is only detectable when acetyl groups are removed from the polyM substrate. This suggests that AlgG epimerization activity in vivo may be affected by prior acetylation of the M residues, or vice versa³⁹. AlgG from *P. fluorescens* also appears to have a signal sequence that would localize it to the periplasm where it presumably acts at the polymer level⁹⁵. Specific mutations that affect the C5-epimerase activity of AlgG in *P. aeruginosa*⁶⁵ and *P. fluorescens*⁴⁹ have recently been identified. Although the *algG4* mutant produces polyM at wild-type levels, an *algG* deletion mutant is drastically affected in polymer production, which is described further below. In addition to the AlgG C-5 epimerase, *A. vinelandii* has other genes for epimerases that can introduce G-blocks (unlike in *Pseudomonas*) and appear to be responsible for the synthesis of complex alginates of various polymeric composition in the cyst capsule¹³⁷.

11. ACETYLATION OF D-MANNURONATE RESIDUES

P. aeruginosa alginate is modified by the addition of acetyl groups to the O-2 and/or O-3 positions by an unknown mechanism^{23, 130}. Localized-mutagenesis of the *algD* operon was used to identify genes required for acetylation⁴⁰. Briefly, a cosmid clone of the *algD* operon was packaged in lambda phage, subjected to saturation chemical mutagenesis, and then used to replace the same chromosomal region in strain FRD1. Acetylated alginate is more resistant to lyases, and so recombinants with defects in alginate acetylation were found by screening for reduced resistance to depolymerization by an alginate lyase. Mutants were then confirmed by a colorimetric assay and infrared spectroscopy for loss of acetyl groups. A merodiploid analysis and transposon mutagenesis located an acetylation gene, which was named *algF*⁴⁰. Two genes are adjacent to *algF*, termed *algI* and *algJ*. All mutants with specific defects in *algI*, *algJ*, or *algF* display mucoid colonies identical to the parent strain, and produce an M+G alginate, but the polymer is nonacetylated, as shown by Fourier transform-infrared spectroscopy⁴⁰.

The cellular locations of AlgI, AlgJ, and AlgF were determined using alkaline phosphatase (*phoA*) fusions constructed to each gene using

progressive exonuclease digestion to determine exposure to the periplasm⁴². All in-frame fusions to *algF* and *algJ* are active, indicating that both localize to the periplasm. N-terminal sequence analysis shows that the signal peptide is removed from AlgF, but not from AlgJ. Also, AlgF is released with the periplasmic contents, while AlgJ remains with spheroplasts, indicating that AlgJ is membrane bound. The mapping of active and inactive *phoA* fusions to *algI* shows that AlgI is a membrane protein with seven *trans*-membrane domains. These results support a model for an alginate O acetylation complex that probably interacts with a hypothetical alginate transport apparatus in the periplasm (Figure 8). AlgI is expected to play a role in the transfer of acetyl groups from an acetyl donor, probably acetyl-coenzyme A ($[H_3C-OC-]-S-CoA$), across the cell membrane to AlgJ and AlgF, which then transfers acetyl groups to M residues. AlgX, which may be part of the alginate transport apparatus in the periplasm, shows homology to AlgJ⁴¹ and so may play a role in this acetylation process.

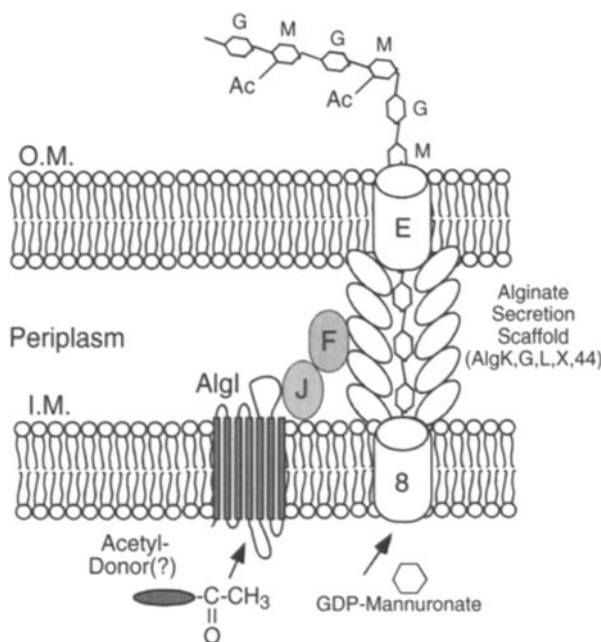


Figure 8. Model for polymerization, transport and acetylation of alginate in *P. aeruginosa*. Alg8 is predicted to be the glycosyl transferase that forms polyM from GDP-mannuronate (synthesized as shown in Figure 7). A putative alginate secretion scaffold, connecting the inner membrane to secretin AlgE in the outer membrane, is predicted to consist of AlgK, AlgG, AlgL, and possibly AlgX and Alg44. AlgG epimerizes no more than every other M residue during export through the periplasm, and is also required to protect the polymer from AlgL's lyase activity⁶⁵. AlgI, AlgJ, and AlgF (shown as dark objects) apparently form a membrane complex that mediates acetylation of the polymer using an unknown acetyl donor, but are not required for polymer formation⁴².

O acetylation is known to affect the physical properties of alginate, including viscosity and interaction with calcium ions¹³², which suggests that alginate O acetylation might play a role in the ability of *P. aeruginosa* to form biofilms in vivo. In CF patients, mucoid *P. aeruginosa* is known to form an unusual biofilm with bacterial microcolonies in the pulmonary tract⁷³. The role of acetylation in biofilm kinetics was examined using mutants with specific acetylation defects⁹⁶. IR spectra of developing biofilms show that mucoid strain FRD1 and its *algT* mutant derivatives form biofilms with rapidly accumulating mass, whereas an *algD* mutant clearly shows a lag during initial biofilm development. Interestingly, an *algJ* mutant defective in the acetylation of alginate produced a very poor biofilm over time. Using scanning confocal laser microscopy and cells containing green fluorescent protein, it was clear that mucoid FRD1 produces microcolonies, whereas nonmucoid derivatives (*algT* and *algD* mutants) produce shallow and densely packed biofilms. In contrast, a mucoid *algJ* mutant demonstrates little attachment and/or surface associated growth. These studies show that the ability to make alginate affects biofilm structure and rate of development, but O acetylation is critical for biofilm formation by a mucoid strain⁹⁶.

Another potential role for alginate O acetylation is related to the fact that CF patients are unable to clear mucoid *P. aeruginosa* despite the elicitation of specific antibodies, which fail to mediate opsonic killing¹⁰⁸. This in part is due to mutations that result in a loss of LPS O side chains as target immunogens, which is common in mucoid *P. aeruginosa* CF strains⁵⁸. However, linking acetate to hydroxyl groups of alginate could permit escape of phagocytic killing by dampening the activation of complement. Evidence for this comes from the observation that small amounts of normal human serum can readily mediate opsonic killing of an *algJ* mutant of FRD1, which forms nonacetylated alginate, even though parent strain FRD1 is resistant¹⁰⁶. CF patients also elicit alginate-specific antibodies, but they are incapable of mediating opsonic killing of *P. aeruginosa*⁹⁰. However, even in very low complement concentrations, the usually nonopsonic alginate-antibodies can readily mediate phagocytic killing of a non-O acetylated mucoid strain¹⁰⁶. Overall, these results indicate a molecular basis for the pathogenesis of chronic mucoid *P. aeruginosa* infection in CF lungs; O acetylation of alginate protects mucoid *P. aeruginosa* from host defenses by preventing activation of the alternative complement pathway, resulting in resistance to antibody-dependent phagocytosis, and renders antibodies that form to alginate to be nonopsonic, which precludes phagocytic killing¹⁰⁶.

12. ALGINATE EXPORT

The *algE* gene encodes a 54-kDa protein with a signal peptide that is cleaved during AlgE export from the cytoplasm¹⁶. Subsequent studies show it

to be in the outer membrane of mucoid bacteria¹¹⁴. Recombinant AlgE incorporates into planar lipid bilayers, probably forming a β -barrel, and it forms an ion channel that is strongly anion selective. Thus, AlgE is likely to be a porin-like protein (e.g., secretin) that is specific for alginate extrusion from the outer membrane of the cell¹¹⁴.

Another gene in the alginate operon that remained uncharacterized until recently was *algK*, between *alg44* and *algE* (see Figure 4), which encodes a 52-kDa protein⁶⁷. The sequence of AlgK shows an apparent signal peptide with an N-terminal cysteine on the mature protein that is characteristic of lipoproteins. AlgK-PhoA fusion proteins are active, indicating that AlgK is periplasmic, and so possibly associated with the outer membrane where it may interact with AlgE. Mutants with a nonpolar *algK* are nonmucoid, indicating that AlgK is essential for polymer formation. The *algK* mutant also demonstrates a small-colony phenotype on L agar, which is suppressed by a polar Tn501 insertion that blocks *algA* expression and hence precursor formation. AlgK⁻ mutants appear to accumulate a toxic extracellular product, which is a low-molecular weight uronic acid, suggesting that AlgK plays an important role in the release of polymerized alginate from the cell⁶⁷.

As described above, AlgG (C5-epimerase) point mutants have been isolated that are deficient in C5-epimerase activity and still form mucoid colonies that contain polyM and lack L-guluronate residues¹⁴. However, a complete deletion of *algG* in *P. aeruginosa*⁶⁵ and *P. fluorescens*⁹⁵ results in a nonmucoid phenotype. Thus, AlgG has another role in alginate biosynthesis besides C5-epimerization. Like an Δ *algK* mutant, an Δ *algG* mutant of *P. aeruginosa* secretes dialyzable uronic acids. An NMR analysis of these secreted uronic acids clearly shows evidence that they are breakdown products of alginate by an alginate lyase⁶⁵. The alginate operon includes *algL* (see Figure 4) which encodes a periplasmic alginate lyase¹²⁰. The ¹H-NMR pattern of the products of alginate degraded by AlgL from *A. vinelandii*, an alginate lyase homologous to AlgL of *P. aeruginosa*, produces a pattern¹¹⁵ that is the same as that from the dialyzable uronic acids secreted by the Δ *algG* mutant of *P. aeruginosa* FRD1⁶⁵. Similar results were also observed in an Δ *algG* mutant of *P. fluorescens*⁴⁹. Thus, AlgG and AlgK probably protect new polymers from degradation by AlgL during transport through the periplasm to the outer membrane⁶⁵. These results suggest that AlgG and AlgK are part of a multi-protein complex that is required for translocation/polymerization of alginate.

13. ROLE OF ALGINATE LYASE, ALGL

Alginate lyases (alginases) are enzymes produced by a wide range of organisms that catalyze the degradation of the alginate polymer into

unsaturated oligosaccharides¹⁴⁵. Most alginate lyase-producing marine bacteria can use alginate from brown algae as a carbon source. However, alginate-producing bacteria like *Pseudomonas* and *Azotobacter* have a periplasmic-localized alginate lyase, but are apparently unable to use alginate as a carbon source. The function(s) of *algL* encoded alginate lyase in alginate producing bacteria has been somewhat controversial. AlgL may control the length of the polymer produced⁸⁸. In support of this, an *algL* mutant was constructed in *A. vinelandii*; it produces an alginate with a higher mean molecular weight than the parent strain, with neither encystment nor cyst germination being affected¹⁴¹. AlgL may be important in facilitating dissemination of bacteria; overexpression of *algL* in mucoid *P. aeruginosa* leads to a decrease in alginate polymer length and an increase in bacterial detachment from an adherent surface¹⁰. With *algL* in the operon for alginate biosynthesis, it is reasonable that a periplasmic AlgL may also be part of a polymerization or transport complex in the periplasm. In *P. syringae* pv. *syringae*, the absence of lyase activity reduces alginate production by about 50%¹⁰³. In *P. aeruginosa*, lyase is reportedly not required for alginate production by *P. aeruginosa*¹¹, yet in another report a lyase-negative *P. aeruginosa* appeared nonmucoid and produced only small amounts of alginate⁹⁴. Recently, we examined the effect of a nonpolar Δ *algL* mutation in mucoid *P. aeruginosa* FRD1, but found such mutants very difficult to construct. By artificially replacing the *algD* operon promoter with a *tac* promoter and IPTG control, Δ *algL* mutants could be constructed when the operon was silent. However, when *algD* operon expression was reactivated with IPTG, the periplasm filled with alginate and the Δ *algL* cells ultimately burst⁶⁶. Thus, AlgL may be a required part of a periplasmic transport apparatus (or scaffold) in mucoid *P. aeruginosa*, along with AlgG and AlgK (Figure 8). A putative role for AlgL lyase activity is to digest periplasmic alginate remaining after spontaneous disassembly of the scaffold.

14. PATHOGENIC MECHANISMS IN MUCOID *P. AERUGINOSA*

As described in the beginning of this chapter, individuals with CF are highly susceptible to bronchiopulmonary infection with strains of *P. aeruginosa* that usually convert to the Alg⁺ form, which is associated with chronic infection⁵⁵. Mutations resulting in defective LPS are also common with such CF strains^{58, 78, 104}, even though LPS is an important virulence factor in acute and systemic infections¹⁸. These “rough” mutations and other alterations and attenuations in multiple virulence factors suggest that CF strains of *P. aeruginosa* have adapted to utilize an alternate set of pathogenic mechanisms that promote chronic lung disease in the CF patient. Unfortunately, the rough LPS-defect

results in low virulence in established animal models, making it difficult to evaluate putative virulence genes in these strains. Thus, in an effort to identify new virulence determinants in CF isolates of *P. aeruginosa*, an alternative model of infection was developed using wounded alfalfa seedlings¹²⁸. Only 1,000 cells of such LPS-defective bacteria can show disease symptoms in the alfalfa infection. Strain FRD1, a typical mucoid CF isolate, and various isogenic mutants have been tested for alfalfa seedling infection. A defect in alginate biosynthesis results in plant disease with >3-fold more bacteria per plant, suggesting that alginate overproduction actually attenuates bacterial growth *in planta*. FRD1 derivatives with *algT(algU)* defects were reduced >50% in the frequency of infection. Thus, σ^{22} apparently regulates factors in mucoid CF strains, besides alginate, that are important for pathogenesis in this model and possibly in the CF lung¹²⁸.

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REFERENCES

1. Baltimore, R.S. and Mitchell, M., 1982, Immunologic investigations of mucoid strains of *Pseudomonas aeruginosa*: Comparison of susceptibility to opsonic antibody in mucoid and nonmucoid strains. *J. Infect. Dis.*, 141:238–247.
2. Banerjee, P.C., Vanags, R.I., Chakrabarty, A.M., and Maitra, P.K., 1983, Alginic acid synthesis in *Pseudomonas aeruginosa* mutants defective in carbohydrate metabolism. *J. Bacteriol.*, 155:238–245.
3. Banerjee, P.C., Vanags, R.I., Chakrabarty, A.M., and Maitra, P.K., 1985, Fructose 1,6-bisphosphate aldolase activity is essential for synthesis of alginate from glucose by *Pseudomonas aeruginosa*. *J. Bacteriol.*, 161:458–460.
4. Bayer, A.S., Park, S., Ramos, M.C., Nast, C.C., Eftekhari, F., and Schiller, N.L., 1992, Effects of alginase on the natural history and antibiotic therapy of experimental endocarditis caused by mucoid *Pseudomonas aeruginosa*. *Infect. Immun.*, 60:3979–3985.
5. Baynham, P. and Wozniak, D., 1996, Identification and characterization of AlgZ, an AlgT-dependent DNA-binding protein required for *Pseudomonas aeruginosa* *algD* transcription. *Mol. Microbiol.*, 22:97–108.
6. Baynham, P.J., Brown, A.L., Hall, L.L., and Wozniak, D.J., 1999, *Pseudomonas aeruginosa* AlgZ, a ribbon-helix-helix DNA-binding protein, is essential for alginate synthesis and *algD* transcriptional activation. *Mol. Microbiol.*, 33:1069–1080.
7. Berry, A., DeVault, J.D., and Chakrabarty, A.M., 1989, High osmolarity is a signal for enhanced *algD* transcription in mucoid and nonmucoid *Pseudomonas aeruginosa* strains. *J. Bacteriol.*, 171:2312–2317.

8. Boucher, J., Schurr, M., Yu, H., Rowen, D., and Deretic, V., 1997, *Pseudomonas aeruginosa* in cystic fibrosis: Role of *mucC* in the regulation of alginate production and stress sensitivity. *Microbiology*, 143:3473–3480.
9. Boucher, J.C., Yu, H., Mudd, M.H., and Deretic, V., 1997, Mucoid *Pseudomonas aeruginosa* in cystic fibrosis: Characterization of *muc* mutations in clinical isolates and analysis of clearance in a mouse model of respiratory infection. *Infect. Immun.*, 65:3838–3846.
10. Boyd, A. and Chakrabarty, A.M., 1994, Role of alginate lyase in cell detachment of *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.*, 60:2355–2359.
11. Boyd, A., Ghosh, M., May, T.B., Shinabarger, D., Keogh, R., and Chakrabarty, A.M., 1993, Sequence of the *algL* gene of *Pseudomonas aeruginosa* and purification of its alginate lyase product. *Gene*, 131:1–8.
12. Brown, R., McBurney, A., Lunee, J., and Kelly, F., 1995, Oxidative damage to DNA in patients with cystic fibrosis. *Free Radic. Biol. Med.*, 18:801–806.
13. Campbell, J.A., Davies, G.J., Bulone, V., and Henrissat, B., 1997, A classification of nucleotide-diphospho-sugar glycosyltransferases based on amino acid sequence similarities. *Biochem. J.*, 326(Pt 3):929–939.
14. Chitnis, C.E. and Ohman, D.E., 1990, Cloning of *Pseudomonas aeruginosa* *algG*, which controls alginate structure. *J. Bacteriol.*, 172:2894–2900.
15. Chitnis, C.E. and Ohman, D.E., 1993, Genetic analysis of the alginate biosynthetic gene cluster of *Pseudomonas aeruginosa* shows evidence of an operonic structure. *Mol. Microbiol.*, 8:583–590.
16. Chu, L., May, T.B., Chakrabarty, A.M., and Misra, T.K., 1991, Nucleotide sequence and expression of the *algE* gene involved in alginate biosynthesis by *Pseudomonas aeruginosa*. *Gene*, 107:1–10.
17. Coyne, M.J., Jr, Russell, K.S., Coyle, C.L., and Goldberg, J.B., 1994, The *Pseudomonas aeruginosa* *algC* gene encodes phosphoglucomutase, required for the synthesis of a complete lipopolysaccharide core. *J. Bacteriol.*, 176:3500–3507.
18. Cryz, Jr, S.J., Fürer, Jr, E., and Germanier, R., 1984, Role of lipopolysaccharide in virulence of *Pseudomonas aeruginosa*. *Infect. Immun.*, 44:508–513.
19. Cryz, S.J., Sadoff, Jr, J.C., Ohman, D., and Fürer, E., 1988, Characterization of the human immune response to a *Pseudomonas aeruginosa* O-polysaccharide-toxin A conjugate vaccine. *J. Lab. Clin. Med.*, 111:701–707.
20. Darzins, A., Frantz, B., Vanags, R.I., and Chakrabarty, A.M., 1986, Nucleotide sequence analysis of the phosphomannose isomerase gene (*pmi*) of *Pseudomonas aeruginosa* and comparison with the corresponding *Escherichia coli* gene *manA*. *Gene*, 42:293–302.
21. Darzins, A., Nixon, L.L., Vanags, R.I., and Chakrabarty, A.M., 1985, Cloning of *Escherichia coli* and *Pseudomonas aeruginosa* phosphomannose isomerase genes and their expression in alginate-negative mutants of *Pseudomonas aeruginosa*. *J. Bacteriol.*, 161:249–257.
22. Darzins, A., Wang, S.-K., Vanags, R.I., and Chakrabarty, A.M., 1985, Clustering of mutations affecting alginic acid biosynthesis in mucoid *Pseudomonas aeruginosa*. *J. Bacteriol.*, 164:516–524.
23. Davidson, J.W., Lawson, C.J., and Sutherland, I.W., 1977, Localization of O-acetyl groups in bacterial alginate. *J. Gen. Microbiol.*, 98:603–606.
24. De Las Penas, A., Connolly, L., and Gross, C.A., 1997, SigmaE is an essential sigma factor in *Escherichia coli*. *J. Bacteriol.*, 179:6862–6864.
25. De Las Penas, A., Connolly, L., and Gross, C.A., 1997, The sigmaE-mediated response to extracytoplasmic stress in *Escherichia coli* is transduced by RseA and RseB, two negative regulators of sigmaE. *Mol. Microbiol.*, 24:373–385.
26. Deretic, V., Dikshit, R., Konyecsni, M., Chakrabarty, A.M., and Misra, T.K., 1989, The *algR* gene, which regulates mucoidy in *Pseudomonas aeruginosa*, belongs to a class of environmentally responsive genes. *J. Bacteriol.*, 171:1278–1283.

27. Deretic, V., Gill, J.F., and Chakrabarty, A.M., 1987, Gene *algD* coding for GDP-mannose dehydrogenase is transcriptionally activated in mucoid *Pseudomonas aeruginosa*. *J. Bacteriol.*, 169:351–358.
28. Deretic, V. and Konyecsni, W.M., 1989, Control of mucoidy in *Pseudomonas aeruginosa*: Transcriptional regulation of *algR* and identification of the second regulatory gene, *algQ*. *J. Bacteriol.*, 171:3680–3688.
29. Deretic, V., Schurr, M.J., Boucher, J.C., Deretic, V., Schurr, M.J., Boucher, J.C., and Martin, D.W., 1994, Conversion of *Pseudomonas aeruginosa* to mucoidy in cystic fibrosis: Environmental stress and regulation of bacterial virulence by alternative sigma factors. *J. Bacteriol.*, 176:2773–2780.
30. DeVault, J.D., Hendrickson, W., Kato, J., and Chakrabarty, A.M., 1991, Environmentally regulated *algD* promoter is responsive to the cAMP receptor protein in *Escherichia coli*. *Mol. Microbiol.*, 5:2503–2509.
31. DeVries, C.A. and Ohman, D.E., 1994, Mucoid to nonmucoid conversion in alginate-producing *Pseudomonas aeruginosa* often results from spontaneous mutations in *algT*, encoding a putative alternative sigma factor, and shows evidence for autoregulation. *J. Bacteriol.*, 176:6677–6687.
32. diSant'Agnese, P.A. and Davis, P.B., 1976, Research in cystic fibrosis. *New Eng. J. Med.*, 295:597–602.
33. Doggett, R.G., Harrison, G.M., Stillwell, R.N., and Wallis, E.S., 1966, An atypical *Pseudomonas aeruginosa* associated with cystic fibrosis of the pancreas. *J. Pediatr.*, 68:215–221.
34. Erickson, J.W. and Gross, C.A., 1989, Identification of the σ^E subunit of *Escherichia coli* RNA polymerase: A second alternate σ factor involved in high-temperature gene expression. *Genes Dev.*, 3:1462–1471.
35. Evans, L.R. and Linker, A., 1973, Production and characterization of the slime polysaccharide of *Pseudomonas aeruginosa*. *J. Bacteriol.*, 116:915–924.
36. Firoved, A.M. and Deretic, V., 2003, Microarray analysis of global gene expression in mucoid *Pseudomonas aeruginosa*. *J. Bacteriol.*, 185:1071–1081.
37. Flynn, J.L. and Ohman, D.E., 1988, Cloning of genes from mucoid *Pseudomonas aeruginosa* which control spontaneous conversion to the alginate production phenotype. *J. Bacteriol.*, 170:1452–1460.
38. Flynn, J.L. and Ohman, D.E., 1988, Use of a gene replacement cosmid vector for cloning alginate conversion genes from mucoid and nonmucoid *Pseudomonas aeruginosa* strains: *algS* controls expression of *algT*. *J. Bacteriol.*, 170:3228–3236.
39. Franklin, M.J., Chitnis, C.E., Gacesa, P., Sonesson, A., White, D.C., and Ohman, D.E., 1994, *Pseudomonas aeruginosa* AlgG is a polymer level alginate C5-mannuronan epimerase. *J. Bacteriol.*, 176:1821–1830.
40. Franklin, M.J. and Ohman, D.E., 1993, Identification of *algF* in the alginate biosynthetic gene cluster of *Pseudomonas aeruginosa* which is required for alginate acetylation. *J. Bacteriol.*, 175:5057–5065.
41. Franklin, M.J. and Ohman, D.E., 1996, Identification of *algI* and *algJ* in the *Pseudomonas aeruginosa* alginate biosynthetic gene cluster which are required for alginate O acetylation. *J. Bacteriol.*, 178:2186–2195.
42. Franklin, M.J. and Ohman, D.E., 2002, Mutant analysis and cellular localization of the AlgI, AlgJ, and AlgF proteins required for O acetylation of alginate in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 184:3000–3007.
43. Frederiksen, B., Koch, C., and Høiby, N., 1997, Antibiotic treatment of initial colonization with *Pseudomonas aeruginosa* postpones chronic infection and prevents deterioration of pulmonary function in cystic fibrosis. *Pediatr. Pulmonol.*, 23:330–335.

44. Fujiwara, S., Zielinski, N.A., and Chakrabarty, A.M., 1993, Enhancer-like activity of AlgR1-binding site in alginate gene activation: Positional, orientational, and sequence specificity. *J. Bacteriol.*, 175:5452–5459.
45. Gacesa, P., 1998, Bacterial alginate biosynthesis—recent progress and future prospects. *Microbiology*, 144(Pt 5):1133–1143.
46. Gacesa, P. and Russell, N.J., 1990, *Pseudomonas Infection and Alginates: Biochemistry, Genetics and Pathology*. Chapman & Hall, London.
47. Gacesa, P. and Russell, N.J., 1990, The structure and properties of alginate. In P. Gacesa and N.J. Russell (eds), *Pseudomonas Infections and Alginates: Biochemistry, Genetics and Pathology*, pp. 29–49. Chapman & Hall Ltd., London.
48. Gill, J.F., Deretic, V., and Chakrabarty, A.M., 1986, Overproduction and assay of *Pseudomonas aeruginosa* phosphomannose isomerase. *J. Bacteriol.*, 167:611–615.
49. Gimmetstad, M., Sletta, H., Ertesvag, H., Bakkevig, K., Jain, S., Suh, S.J., Skjak-Braek, G., Ellingsen, T.E., Ohman, D.E., and Valla, S., 2003, The *Pseudomonas fluorescens* AlgG protein, but not its mannuronan C-5-epimerase activity, is needed for alginate polymer formation. *J. Bacteriol.*, 185:3515–3523.
50. Goldberg, J.B., Gorman, W.L., Flynn, J.L., and Ohman, D.E., 1993, A mutation in *algN* permits trans-activation of alginate production by *algT* in *Pseudomonas* species. *J. Bacteriol.*, 175:1303–1308.
51. Goldberg, J.B., Hatano, K., and Pier, G.B., 1993, Synthesis of lipopolysaccharide O side chains by *Pseudomonas aeruginosa* PAO1 requires the enzyme phosphomannomutase. *J. Bacteriol.*, 175:1605–1611.
52. Goldberg, J.B. and Ohman, D.E., 1984, Cloning and expression in *Pseudomonas aeruginosa* of a gene involved in the production of alginate. *J. Bacteriol.*, 158:1115–1121.
53. Goldberg, J.B. and Ohman, D.E., 1987, Construction and characterization of *Pseudomonas aeruginosa algB* mutants: Role of *algB* in high-level production of alginate. *J. Bacteriol.*, 169:1593–1602.
54. Gorin, P.A.T. and Spencer, J.F.T., 1966, Exocellular alginic acid from *Azotobacter vinelandii*. *Can. J. Chem.*, 44:993–998.
55. Govan, J.R., Martin, D.W., and Deretic, V., 1992, Mucoid *Pseudomonas aeruginosa* and cystic fibrosis: The role of mutations in *muc* loci. *FEMS Microbiol. Lett.*, 79:323–329.
56. Govan, J.R.W. and Deretic, V., 1996, Microbial pathogenesis in cystic fibrosis: Mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol. Rev.*, 60:539–574.
57. Govan, J.R.W. and Harris, G.S., 1986, *Pseudomonas aeruginosa* and cystic fibrosis: Unusual bacterial adaptation and pathogenesis. *Microbiol. Sci.*, 3:302–308.
58. Hancock, R.E.W., Mutharia, L.M., Chan, L., Darveau, R.P., Speert, D.P., and Pier, G.B., 1983, *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis: A class of serum-sensitive, nontypable strains deficient in lipopolysaccharide O side chains. *Infect. Immun.*, 42:170–177.
59. Hassett, D.J., Charniga, L., Bean, K., Ohman, D.E., and Cohen, M.S., 1992, Response of *Pseudomonas aeruginosa* to pyocyanin: Mechanisms of resistance, antioxidant defenses, and demonstration of a manganese-cofactored superoxide dismutase. *Infect. Immun.*, 60:328–336.
60. Hassett, D.J., Woodruff, W.A., Wozniak, D.J., Vasil, M., Cohen, M.S., and Ohman, D.E., 1993, Cloning and characterization of *Pseudomonas aeruginosa sodB* and *sodA* genes encoding iron- and manganese-cofactored superoxide dismutase: Demonstration of increase manganese superoxide dismutase activity in alginate-producing bacteria. *J. Bacteriol.*, 175:7658–7665.
61. Hershberger, C.D., Ye, R.W., Parsek, M.R., Xie, Z., and Chakrabarty, A.M., 1995, The *algU* (*algU*) gene of *Pseudomonas aeruginosa*, a key regulator involved in alginate biosynthesis, encodes an alternative σ factor (σ^E). *Proc. Natl. Acad. Sci. USA*, 92:7941–7945.
62. Hoiby, N. and Olling, S., 1977, *Pseudomonas aeruginosa* infection in cystic fibrosis: Bactericidal effect of serum from normal individuals and patients with cystic fibrosis on

- P. aeruginosa* strains from patients with cystic fibrosis or other diseases. *Acta Pathol. Microbiol. Scand., Section C*, 85:107–114.
63. Høidal, H.K., Ertesvåg, H., Skjak-Bræk, G., Stokke, B.T., and Valla, S., 1999, The recombinant *Azotobacter vinelandii* mannuronan C-5-epimerase AlgE4 epimerizes alginate by a nonrandom attack mechanism. *J. Biol. Chem.*, 274:12316–12322.
 64. Hull, J., Vervaart, P., Grimwood, K., and Phelan, P., 1997, Pulmonary oxidative stress response in young children with cystic fibrosis. *Thorax*, 52:557–560.
 65. Jain, S., Franklin, M.J., Ertesvåg, H., Valla, S., and Ohman, D.E., 2003, The dual roles of AlgG in C-5-epimerization and secretion of alginate polymers in *Pseudomonas aeruginosa*. *Mol. Microbiol.*, 47:1123–1133.
 66. Jain, S. and Ohman, D.E., 2004, AlgL is essential for the secretion of alginate in mucoid *Pseudomonas aeruginosa*, in preparation.
 67. Jain, S. and Ohman, D.E., 1998, Deletion of *algK* in mucoid *Pseudomonas aeruginosa* blocks alginate polymer formation and results in uronic acid secretion. *J. Bacteriol.*, 180:634–641.
 68. Jensen, E., Kharazmi, A., Lam, K., Costerton, J., and Høiby, N., 1990, Human polymorphonuclear leukocyte response to *Pseudomonas aeruginosa* grown in biofilms. *Infect Immun.*, 58:2383–2385.
 69. Kato, J. and Chakrabarty, A.M., 1991, Purification of the regulatory protein AlgR1 and its binding in the far upstream region of the *algD* promoter in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA*, 88:1760–1764.
 70. Kato, J., Misra, T.K., and Chakrabarty, A.M., 1990, AlgR3, a protein resembling eukaryotic histone H1, regulates alginate synthesis in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA*, 87:2887–2891.
 71. Kim, H., Schlichtman, D., Shankar, S., Xie, Z., Chakrabarty, A., and Kornberg, A., 1998, Alginate, inorganic polyphosphate, GTP and ppGpp synthesis co-regulated in *Pseudomonas aeruginosa*: Implications for stationary phase survival and synthesis of RNA/DNA precursors. *Mol. Microbiol.*, 27:717–725.
 72. Koch, C. and N. Høiby. 1993, Pathogenesis in cystic fibrosis. *Lancet*, 341:1065–1069.
 73. Lam, J., Chan, R., Lam, K., and Costerton, J.R.W., 1980, Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. *Infect. Immun.*, 28:546–556.
 74. Lin, Y.-T. and Hassid, W.Z., 1966, Pathway of alginic acid synthesis in the marine brown alga, *Fucus gardneri* Silva. *J. Biol. Chem.*, 241:5284–5297.
 75. Linker, A. and Jones, R.S., 1966, A new polysaccharide resembling alginic acid isolated from *Pseudomonads*. *J. Biol. Chem.*, 241:3845–3851.
 76. Lloret, L., Barreto, R., Leon, R., Moreno, S., Martinez-Salazar, J., Espin, G., and Soberon-Chavez, G., 1996, Genetic analysis of the transcriptional arrangement of *Azotobacter vinelandii* alginate biosynthetic genes: Identification of two independent promoters. *Mol. Microbiol.*, 21:449–457.
 77. Lonetto, M.A., Brown, K.L., Rudd, K.E., and Buttner, M.J., 1994, Analysis of *Streptomyces coelicolor* *SigE* gene reveals the existence of a subfamily of eubacterial RNA polymerase σ factors involved in the regulation of extracytoplasmic functions. *Proc. Natl. Acad. Sci. USA*, 91:7573–7577.
 78. Luzar, M.A. and Montie, T.C., 1985, Avirulence and altered physiological properties of cystic fibrosis strains of *Pseudomonas aeruginosa*. *Infect. Immun.*, 50:572–576.
 79. Ma, S., Selvaraj, U., Ohman, D.E., Quarless, R., Hassett, D.J., and Wozniak, D.J., 1998, Phosphorylation-independent activity of the response regulators AlgB and AlgR in promoting alginate biosynthesis in mucoid *Pseudomonas aeruginosa*. *J. Bacteriol.*, 180:956–968.
 80. Ma, S., Wozniak, D.J., and Ohman, D.E., 1997, Identification of the histidine protein kinase KinB in *Pseudomonas aeruginosa* and its phosphorylation of the alginate regulator AlgB. *J. Biol. Chem.*, 272:17952–17960.

81. Malhotra, S., Silo-Suh, L.A., Mathee, K., and Ohman, D.E., 2000, Proteome analysis of the effect of mucoid conversion on global protein expression in *Pseudomonas aeruginosa* strain PAO1 shows induction of the disulfide bond isomerase, DsbA. *J. Bacteriol.*, 182:6999–7006.
82. Marcus, H. and Baker, N.R., 1985, Quantitation of adherence of mucoid and nonmucoid *Pseudomonas aeruginosa* to hamster tracheal epithelium. *Infect. Immun.*, 47:723–729.
83. Martin, D.W., Schurr, M.J., Mudd, M.H., and Deretic, V., 1993, Differentiation of *Pseudomonas aeruginosa* into the alginate-producing form: Inactivation of mucB causes conversion to mucoidy. *Mol. Microbiol.*, 9:497–506.
84. Martin, D.W., Schurr, M.J., Mudd, M.H., Govan, J.R.W., Holloway, B.W., and Deretic, V., 1993, Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. *Proc. Natl. Acad. Sci. USA*, 90:8377–8381.
85. Martinez-Salazar, J.M., Moreno, S., Najera, R., Boucher, J.C., Espin, G., Soberon-Chavez, G., and Deretic, V., 1996, Characterization of the genes coding for the putative sigma factor AlgU and its regulators MucA, MucB, MucC, and MucD in *Azotobacter vinelandii* and evaluation of their roles in alginate biosynthesis. *J. Bacteriol.*, 178:1800–1808.
86. Mathee, K., Ciofu, O., Sternberg, C.K., Lindum, P., Campbell, J., Jensen, P., Johnsen, A., Givskov, M., Ohman, D., Molin, S., Høiby, N., and Kharazmi, A., 1999, Mucoid conversion of *Pseudomonas aeruginosa* by hydrogen peroxide: A mechanism for virulence activation in the cystic fibrosis lung. *Microbiology*, 145:1349–1357.
87. Mathee, K., McPherson, C.J., and Ohman, D.E., 1997, Posttranslational control of the *algT* (*algU*)-encoded σ^{22} for expression of the alginate regulon in *Pseudomonas aeruginosa* and localization of its antagonist proteins MucA and MucB (AlgN). *J. Bacteriol.*, 179:3711–3720.
88. May, T.B. and Chakrabarty, A.M., 1994, *Pseudomonas aeruginosa*: Genes and enzymes of alginate synthesis. *Trends Microbiol.*, 2:151–157.
89. May, T.B., Shinabarger, D., Maharaj, R., Kato, J., Chu, L., DeVault, J.D., Roychoudhury, S., Zielinski, N.A., Berry, A., Rothmel, R.K., Misra, T.K., and Chakrabarty, A.M., 1991, Alginate synthesis by *Pseudomonas aeruginosa*: A key pathogenic factor in chronic pulmonary infections of cystic fibrosis patients. *Clin. Microbiol. Rev.*, 4:191–206.
90. Meluleni, G.J., Grout, M., Evans, D.J., and Pier, G.B., 1995, Mucoid *Pseudomonas aeruginosa* growing in a biofilm in vitro are killed by opsonic antibodies to the mucoid exopolysaccharide capsule but not by antibodies produced during chronic lung infection in cystic fibrosis patients. *J. Immunol.*, 155:2029–2038.
91. Missiakas, D., Mayer, M.P., Lemaire, M., Georgopoulos, C., and Raina, S., 1997, Modulation of the *Escherichia coli* sigmaE (RpoE) heat-shock transcription-factor activity by the RseA, RseB and RseC proteins. *Mol. Microbiol.*, 24:355–371.
92. Missiakas, D. and Raina, S., 1998, The extracytoplasmic function sigma factors: Role and regulation. *Mol. Microbiol.*, 28:1059–1066.
93. Mohr, C.D., Rust, L., Albus, A.M., Igilewski, B.H., and Deretic, V., 1990, Expression patterns of genes encoding elastase and controlling mucoidy – Co-ordinate regulation of two virulence factors in *Pseudomonas aeruginosa* isolates from cystic fibrosis. *Mol. Microbiol.*, 4:2103–2110.
94. Monday, S.R. and Schiller, N.L., 1996, Alginate synthesis in *Pseudomonas aeruginosa*: The role of AlgL (alginate lyase) and AlgX. *J. Bacteriol.*, 178:625–632.
95. Morea, A., Mathee, K., Franklin, M.J., Giacomini, A., O'Regan, M., and Ohman, D.E., 2001, Characterization of *algG* encoding C5-epimerase in the alginate biosynthetic gene cluster of *Pseudomonas fluorescens*. *Gene*, 278:107–114.
96. Nivens, D.E., Ohman, D.E., Williams, J., and Franklin, M.J., 2001, Role of alginate and its O acetylation in formation of *Pseudomonas aeruginosa* microcolonies and biofilms. *J. Bacteriol.*, 183:1047–1057.
97. Ohman, D.E. and Chakrabarty, A.M., 1981, Genetic mapping of chromosomal determinants for the production of the exopolysaccharide alginate in a *Pseudomonas aeruginosa* cystic fibrosis isolate. *Infect. Immun.*, 33:142–148.

98. Ohman, D.E. and Chakrabarty, A.M., 1982, Utilization of human respiratory secretions by mucoid *Pseudomonas aeruginosa* of cystic fibrosis origin. *Infect. Immun.*, 37:662–669.
99. Ohman, D.E., Mathee, K., McPherson, C.J., DeVries, C.A., Ma, S., Wozniak, D.J., and Franklin, M.J., 1996, Regulation of the alginate (*algD*) operon in *Pseudomonas aeruginosa*. In T. Nakazawa, K. Furukawa, D. Haas, and S. Silver (eds), *Molecular Biology of Pseudomonads*, pp. 472–483. American Society for Microbiology Press, Washington, DC.
100. Olvera, C., Goldberg, J.B., Sanchez, R., and Soberon-Chavez, G., 1999, The *Pseudomonas aeruginosa algC* gene product participates in rhamnolipid biosynthesis. *FEMS Microbiol. Lett.*, 179:85–90.
101. Pedersen, S.S., 1992, Lung infection with alginate-producing, mucoid *Pseudomonas aeruginosa* in cystic fibrosis. *APMIS* (Suppl.) 28:1–79.
102. Pedersen, S.S., Moller, H., Espersen, F., Sørensen, C.H., Jensen, T., and Høiby, N., 1992, Mucosal immunity to *Pseudomonas aeruginosa* alginate in cystic fibrosis. *Acta Pathol. Microbiol. Immunol. Scand.*, 100:326–334.
103. Penaloza-Vazquez, A., Kidambi, S.P., Chakrabarty, A.M., and Bender, C.L., 1997, Characterization of the alginate biosynthetic gene cluster in *Pseudomonas syringae* pv. *syringae*. *J. Bacteriol.*, 179:4464–4472.
104. Penketh, A.R.L., Wise, A., Mears, M.B., Hodson, M.E., and Batten, J.C., 1987, Cystic fibrosis in adolescents and adults. *Thorax*, 42:526–532.
105. Pier, G.B., 1985, Pulmonary disease associated with *Pseudomonas aeruginosa* in cystic fibrosis: Current status of the host–bacterium interaction. *J. Infect. Dis.*, 151:575–580.
106. Pier, G.B., Coleman, F., Grout, M., Franklin, M., and Ohman, D.E., 2001, Role of alginate O acetylation in resistance of mucoid *Pseudomonas aeruginosa* to opsonic phagocytosis. *Infect. Immun.*, 69:1895–1901.
107. Pier, G.B., Grouot, M., Zaidi, T.S., Olsen, J.C., Johnaon, L.G., Yankaskas, J.R., and Goldberg, J.B., 1996, Role of mutant CFTR in hypersusceptibility of cystic fibrosis to lung infections. *Science*, 271:64–67.
108. Pier, G.B., Saunders, J.M., Ames, P., Edwards, M.S., Auerbach, H., Goldfarb, J., Speert, D.P., and Hurwitch, S., 1987, Opsonophagocytic killing antibody to *Pseudomonas aeruginosa* mucoid exopolysaccharide in older noncolonized patients with cystic fibrosis. *N. Engl. J. Med.*, 317:793–798.
109. Pier, G.B., Small, G.J., and Warren, H.B., 1990, Protection against mucoid *Pseudomonas aeruginosa* in rodent models of endobronchial infections. *Science*, 249:537–540.
110. Piggot, N.H., Sutherland, I.W., and Jarman, T.R., 1981, Enzymes involved in the biosynthesis of alginate by *Pseudomonas aeruginosa*. *Eur. J. Appl. Microbiol. Biotechnol.*, 13:179–183.
111. Pindar, D.F. and Bucke, C., 1975, The biosynthesis of alginic acid by *Azotobacter vinelandii*. *Biochem. J.*, 152:617–622.
112. Pogliano, J., Lynch, A.S., Belin, D., Lin, E.C., and Beckwith, J., 1997, Regulation of *Escherichia coli* cell envelope proteins involved in protein folding and degradation by the Cpx two-component system. *Genes Dev.*, 11:1169–1182.
113. Ramphal, R., Guay, C., and Pier, G.B., 1987, *Pseudomonas aeruginosa* adhesins for tracheo-bronchial mucin. *Infect. Immun.*, 55:600–603.
114. Rehm, B.H., Boheim, G., Tommassen, J., and Winkler, U.K., 1994, Overexpression of *algE* in *Escherichia coli*: Subcellular localization, purification, and ion channel properties. *J. Bacteriol.*, 176:5639–5647.
115. Rehm, B.H., Ertesvåg, H., and Valla, S., 1996, A new *Azotobacter vinelandii* mannuronan C-5-epimerase gene (*algG*) is part of an *alg* gene cluster physically organized in a manner similar to that in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 178:5884–5889.
116. Roehl, R.A., Feary, T.W., and Phibbs, P.V., 1983, Clustering of mutations affecting central pathway enzymes of carbohydrate catabolism in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 156:1123–1129.

117. Rouviere, P.E., De Las Penas, A., Mecsas, J., Lu, C.Z., Rudd, K.E., and Gross, C.A., 1995, *rpoE*, the gene encoding the second heat-shock sigma factor, σ^E , in *Escherichia coli*. *EMBO J.*, 14:1032–1042.
118. Roychoudhury, S., May, T., Gill, J., Singh, S., Feingold, D., and Chakrabarty, A., 1989, Purification and characterization of guanosine diphospho-D-mannose dehydrogenase. A key enzyme in the biosynthesis of alginate by *Pseudomonas aeruginosa*. *J. Biol. Chem.*, 264:9380–9385.
119. Saxena, I.M., Brown, R.M., Fevre, M., Geremia, R.A., and Henrissat, B., 1995, Multidomain architecture of β -glycosyl transferases: Implications for mechanism of action. *J. Bacteriol.*, 177:1419–1424.
120. Schiller, N.L., Monday, S.R., Boyd, C.M., Keen, N.T., and Ohman, D.E., 1993, Characterization of the *Pseudomonas aeruginosa* alginate lyase gene (*algL*): Cloning, sequencing, and expression in *Escherichia coli*. *J. Bacteriol.*, 175:4780–4789.
121. Schurr, M.J., Martin, D.W., Mudd, M.H., and Deretic, V., 1994, Gene cluster controlling conversion of alginate-overproducing phenotype in *Pseudomonas aeruginosa*: Functional analysis in a heterologous host and role in the instability of mucoidy. *J. Bacteriol.*, 176:3375–3382.
122. Schurr, M.J., Yu, H., Martinez-Salazar, J.M., Boucher, J.C., and Deretic, V., 1996, Control of AlgU, a member of the sigma E-like family of stress sigma factors, by the negative regulators MucA and MucB and *Pseudomonas aeruginosa* conversion to mucoidy in cystic fibrosis. *J. Bacteriol.*, 178:4997–5004.
123. Schwarzmann, S. and Boring III, J.R., 1971, Antiphagocytic effect of slime from a mucoid strain of *Pseudomonas aeruginosa*. *Infect. Immun.*, 3:762–767.
124. Seale, T.W., Thirkhill, H., Tarpay, M., Flux, M., and Rennert, O.M., 1979, Serotypes and antibiotic susceptibilities of *Pseudomonas aeruginosa* isolates from single sputa of cystic fibrosis patients. *J. Clin. Microbiol.*, 9:72–78.
125. Sferra, T.J. and Collins, F.S., 1993, The molecular biology of cystic fibrosis. *Annu. Rev. Med.*, 44:133–144.
126. Sherbrock-Cox, V., Russell, N.J., and Gacesa, P., 1984, The purification and chemical characterisation of the alginate present in extracellular material produced by mucoid strains of *Pseudomonas aeruginosa*. *Carbohydr. Res.*, 135:147–154.
127. Shinabarger, D., Berry, A., May, T.B., Rothmel, R., Fialho, A., and Chakrabarty, A.M., 1991, Purification and characterization of phosphomannose isomerase-guanosine diphospho-D-mannose pyrophosphorylase—a bifunctional enzyme in the alginate biosynthetic pathway of *Pseudomonas aeruginosa*. *J. Biol. Chem.*, 266:2080–2088.
128. Silo-Suh, L., Suh, S.J., Sokol, P.A., and Ohman, D.E., 2002, A simple alfalfa seedling infection model for *Pseudomonas aeruginosa* strains associated with cystic fibrosis shows AlgT (sigma-22) and RhlR contribute to pathogenesis. *Proc. Natl. Acad. Sci. USA*, 99:15699–15704.
129. Simpson, J.A., Smith, S.E., and Dean, R.T., 1989, Scavenging by alginate of free radicals released by macrophages. *Ree Radical Biol. Med.*, 6:347–353.
130. Skjåk-Bræk, G., Grasdalen, H., and Larsen, B., 1986, Monomer sequence and acetylation pattern in some bacterial alginates. *Carbohydr. Res.*, 154:239–250.
131. Skjåk-Bræk, G., Larsen, B., and Grasdalen, H., 1985, The role of O-acetyl groups in the biosynthesis of alginate by *Azotobacter vinelandii*. *Carbohydr. Res.*, 145:169–174.
132. Skjåk-Bræk, G., Zanetti, F., and Paoletti, S., 1989, Effect of acetylation on some solution and gelling properties of alginates. *Carbohydr. Res.*, 185:131–138.
133. Smith, J., Travis, S., Greenber, E., and Welsh, M., 1996, Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. *Cell*, 85:229–236.
134. Snook, C.F., Tipton, P.A., and Beamer, L.J., 2003, Crystal structure of GDP-mannose dehydrogenase: A key enzyme of alginate biosynthesis in *P. aeruginosa*. *Biochemistry*, 42:4658–4668.

135. Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrenner, P., Hickey, M.J., Brinkman, F.S.L., Hufnagle, W.O., Kowalik, D.J., Lagrou, M., Garber, R.L., Goltry, L., Tolentino, E., Westbrook-Wadman, S., Yuan, Y., Brody, L.L., Coulter, S.N., Folger, K.R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G.K.-S., Wu, Z., Paulsen, I.T., Reizer, J., Saier, M.H., Hancock, R.E.W., Lory, S., and Olson, M.V., 2000, Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature*, 959:959–964.
136. Suh, S.-J., Silo-Suh, L., Woods, D., Hassett, D., West, S., and Ohman, D., 1999, Effect of *rpoS* mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 181:3890–3897.
137. Svanem, B.I., Skjåk-Bræk, G., Ertesvag, H., and Valla, S., 1999, Cloning and expression of three new *Azotobacter vinelandii* genes closely related to a previously described gene family encoding mannuronan C-5-epimerases. *J. Bacteriol.*, 181:68–77.
138. Tatnell, P.J., Russell, N.J., and Gacesa, P., 1994, GDP-mannose dehydrogenase is the key regulatory enzyme in alginate biosynthesis in *Pseudomonas aeruginosa*: Evidence from metabolite studies. *Microbiology*, 140(Pt 7):1745–1754.
139. Tatnell, P.J., Russell, N.J., and Gacesa, P., 1993, A metabolic study of the activity of GDP-mannose dehydrogenase and concentrations of activated intermediates of alginate biosynthesis in *Pseudomonas aeruginosa*. *J. Gen. Microbiol.*, 139:119–127.
140. Thomassen, M.J., Demko, C.A., Boserbaum, B., Stern, R.C., and Kuchenbrod, P.T., 1979, Multiple isolates of *Pseudomonas aeruginosa* with differing antimicrobial susceptibility patterns from patients with cystic fibrosis. *J. Infect. Dis.*, 140:873–880.
141. Trujillo-Roldan, M.A., Moreno, S., Segura, D., Galindo, E., and Espin, G., 2003, Alginate production by an *Azotobacter vinelandii* mutant unable to produce alginate lyase. *Appl. Microbiol. Biotechnol.*, 60:733–737.
142. Vazquez, A., Moreno, S., Guzman, J., Alvarado, A., and Espin, G., 1999, Transcriptional organization of the *Azotobacter vinelandii* *algGXLVIFA* genes: Characterization of *algF* mutants. *Gene*, 232:217–222.
143. Whitchurch, C.B., Alm, R.A., and Mattick, J.S., 1996, The alginate regulator AlgR and an associated sensor FimS are required for twitching motility in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA*, 93:9839–9843.
144. Whitchurch, C.B., Erova, T.E., Emery, J.A., Sargent, J.L., Harris, J.M., Semmler, A.B., Young, M.D., Mattick, J.S., and Wozniak, D.J., 2002, Phosphorylation of the *Pseudomonas aeruginosa* response regulator AlgR is essential for type IV fimbria-mediated twitching motility. *J. Bacteriol.*, 184:4544–4554.
145. Wong, T.Y., Preston, L.A., and Schiller, N.L., 2000, ALGINATE LYASE: Review of major sources and enzyme characteristics, structure-function analysis, biological roles, and applications. *Annu. Rev. Microbiol.*, 54:289–340.
146. Wood, L.F. and Ohman, D.E., 2004, Identification of an independent promoter for expression of MucD and its role as an HtrA-like protease in *Pseudomonas aeruginosa*, manuscript in preparation.
147. Woods, D.E., Sokol, P.A., Bryan, L.E., Storey, D.G., Mattingly, S.J., Vogel, H.J., and Ceri, H., 1991, In vivo regulation of virulence in *Pseudomonas aeruginosa* associated with genetic rearrangement. *J. Infect. Dis.*, 163:143–149.
148. Woolwine, S. and Wozniak, D., 1999, Identification of an *Escherichia coli* *pepA* homolog and its involvement in suppression of the *algB* phenotype in mucoid *Pseudomonas aeruginosa*. *J. Bacteriol.*, 181:107–116.
149. Wozniak, D.J. and Ohman, D.E., 1993, Involvement of the alginate *algT* gene and integration host factor in the regulation of the *Pseudomonas aeruginosa* *algB* gene. *J. Bacteriol.*, 175:4145–4153.

150. Wozniak, D.J. and Ohman, D.E., 1991, *Pseudomonas aeruginosa* AlgB, a two-component response regulator of the NtrC family, is required for *algD* transcription. *J. Bacteriol.*, 173:1406–1413.
151. Wozniak, D.J. and Ohman, D.E., 1994, Transcriptional analysis of the *Pseudomonas aeruginosa* genes *algR*, *algB*, and *algD* reveals a hierarchy of alginate gene expression which is modulated by *algT*. *J. Bacteriol.*, 176:6007–6014.
152. Wyckoff, T.J., Thomas, B., Hassett, D.J., and Wozniak, D.J., 2002, Static growth of mucoid *Pseudomonas aeruginosa* selects for non-mucoid variants that have acquired flagellum-dependent motility. *Microbiology*, 148:3423–3430.
153. Yu, H., Mudd, M., Boucher, J.C., Schurr, M.J., and Deretic, V., 1997, Identification of the *algZ* gene upstream of the response regulator *algR* and its participation in control of alginate production in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 179:187–193.
154. Yu, H., Schurr, M.J., and Deretic, V., 1995, Functional equivalence of *Escherichia coli* σ^E and *Pseudomonas aeruginosa* AlgU: *E. coli* *rpoE* restores mucoidy and reduces sensitivity to reactive oxygen intermediates in *algU* mutants of *P. aeruginosa*. *J. Bacteriol.*, 177:3259–3268.

FATTY ACID BIOSYNTHESIS AND BIOLOGICALLY SIGNIFICANT ACYL TRANSFER REACTIONS IN PSEUDOMONADS

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1. INTRODUCTION

Fatty acid synthesis (FAS) is essential for cellular function by providing precursors for synthesis of numerous cellular constituents, including phospholipids¹⁶, lipopolysaccharides⁸⁴, rhamnolipids^{9, 72}, polyhydroxyalkanoic acids^{2, 61}, oligosaccharides^{23, 27} and proteins⁴⁸. More recently, acyl-acyl carrier proteins (acyl-ACPs) derived from the fatty acid biosynthetic (Fab) pathway were shown to be the acyl donors for synthesis of acylated homoserine lactones (AHLs) that are required for cell-to-cell communication and the many processes depending on it^{41, 43, 67, 76, 100}. In addition, fatty acids play numerous other diverse roles in the physiology of pseudomonads. Adaptive alterations of membrane fatty acids and phospholipids play a role in the solvent tolerance of *Pseudomonas putida* and probably other pseudomonads^{37, 86}. Such adaptive fatty acid alterations also occur in response to changes in growth conditions, with temperature and growth phase being the most influential parameters in *Pseudomonas aeruginosa*²¹. Fatty acid composition of phospholipids also can influence some behavioral traits, such as *P. aeruginosa* twitching motility up

phospholipid gradients⁵³. The unique fatty acid patterns of individual *Pseudomonas* strains determined by gas chromatographic analysis can be utilized to identify individual bacterial species^{19, 68, 69}. This technology forms the basis of the commercially available Sherlock® microbial identification system (MIDI, Inc., Newark, Delaware, USA).

Despite the importance that fatty acids play in the physiology and virulence of many pseudomonads, the genetics and molecular biology of pseudomonal fatty acid biosynthesis and its linked pathways have mostly been elucidated in *P. aeruginosa*. While this chapter will in many parts focus on this bacterium, it can be anticipated that the general pathways will be similar in other pseudomonads and related bacteria, with the exception of the synthesis of some unique cellular constituents that most likely will require some unique enzymes not found in *P. aeruginosa*. Wherever these unique aspects are known, they are described in this chapter.

2. ENZYMES AND PROTEINS OF *PSEUDOMONAS* FATTY ACID SYNTHESIS

Fatty acid biosynthetic pathways typically exist in two distinct molecular forms called types I and II. In type I pathways, the active sites catalyzing the diverse biosynthetic functions are found in distinct domains of large multifunctional proteins, either in a single protein (as in mammals and mycobacteria) or divided between two interacting proteins (as in fungi and *Corynebacterium ammoniagenes*)^{4, 8}. In contrast, each catalytic activity involved in type II FAS systems is contained in a discrete protein^{8, 16}. In most bacteria, including *P. aeruginosa*, the genes encoding bacterial FAS enzymes can either be clustered or encoded by single genes (Table 1). The extremes with regard to genetic organization are *Aquifex aeolicus*, in which only two *fab* genes are adjacent and *Clostridium acetobutylicum*, in which the entire pathway of saturated FAS is contained in a single gene cluster⁸.

Fatty acid synthesis can be roughly divided into two phases, the initiation phase and the elongation phase (Figure 1). These phases have been best characterized in *E. coli*⁸, and genetic and in vitro evidence indicates that similar reactions operate in *P. aeruginosa*^{41-43, 56}. In the initiation phase, the two-carbon molecule acetyl-coenzyme A (acetyl-CoA) is converted to the three-carbon molecule malonyl-CoA in the ATP-dependent acetyl carboxylase reaction. The malonyl group is then transacylated onto ACP by FabD in a malonyl-CoA:ACP transacylation reaction. During the elongation phase, the malonyl-ACP is condensed by one of several condensation enzymes with either an acetyl-CoA (1st round of elongation; thought to be catalyzed by FabH) or malonyl-ACP (subsequent rounds of elongation; catalyzed by FabB and FabF) to form a β -ketoacyl-ACP, which then undergoes consecutive

Table 1. Genes and enzymes of *P. aeruginosa* FAS and modification.

Genes and enzyme function or pathway	PA number ¹	Gene arrangement	References
<i>Acetyl-CoA carboxylase</i>			
<i>accA</i> Carboxyl-transferase subunit	PA3639	Alone	[5]
<i>accB</i> Biotin carboxylase carrier protein	PA4847	<i>accB accC</i>	[5]
<i>accC</i> Biotin carboxylase	PA4848	<i>accB accC</i>	[5]
<i>accD</i> Carboxyl-transferase subunit	PA3112	Alone	[5]
<i>Saturated FAS</i>			
<i>acpP</i> Apo-acyl carrier protein (apo-ACP)	PA2966	<i>fab</i> cluster	[56]
<i>fabD</i> Malonyl-CoA:ACP transacylase	PA2968	<i>fab</i> cluster	[56]
<i>fabF1</i> β -ketoacyl-ACP synthase II	PA2965	<i>fab</i> cluster	[56]
<i>fabG</i> β -ketoacyl-ACP reductase	PA2967	<i>fab</i> cluster	[56]
<i>fabI</i> Enoyl-ACP reductase I	PA1806	Last gene in an unrelated oligopeptide permease-encoding operon	[41]
<i>fabK</i> Enoyl-ACP reductase II	?	?	
<i>fabZ</i> β -hydroxyacyl-ACP dehydrase	PA3645	Lipid A cluster	[43]
<i>Unsaturated FAS</i>			
<i>fabA</i> β -hydroxyacyl-ACP dehydrase/isomerase	PA1610	<i>fabA fabB</i>	[42]
<i>fabB</i> β -ketoacyl-ACP synthase I	PA1609	<i>fabA fabB</i>	[42]
<i>Peripheral enzymes</i>			
<i>pcpS</i> ACP synthase	PA1165	Alone	[25]
<i>rhlG</i> β -ketoacyl reductase	PA3387	Alone	[9]
<i>phaG</i> β -hydroxyacyl-ACP:CoA transacylase	PA0730	Alone	[45]
<i>birA</i> Biotin ligase	PA4280	Alone	
<i>Potential other FAS/modifying enzymes</i>			
<i>fabF2</i> β -ketoacyl-ACP synthase II	PA1373	Alone	
Acyl carrier protein	PA1869	Alone	
Acyl carrier protein	PA3334	PA3330-PA3334 cluster?	
Fatty acid desaturase	PA0286	Alone	
<i>Cis-trans</i> isomerase	PA1846	Alone	
Long chain acyl-CoA synthase	PA2893	Cluster with other genes	
<i>fabH2</i> Probable 3-oxo-acyl-ACP synthase III	PA3333	PA3330-PA3334 cluster?	
Medium chain acyl-CoA ligase	PA3924	Clustered with PA3925?	
Acyl-CoA thiolase	PA3925	Clustered with PA3924?	
<i>tesB</i> Acyl-CoA thioesterase	PA3942	Alone	
β -ketoacyl synthase	PA5174	Alone	

¹Gene annotations and open reading frame (PA) numbers are taken from the annotated PAO1 genome sequence⁹⁸ (www.pseudomonas.com).

reduction, dehydration and reduction reactions to complete each round of chain elongation, resulting in acyl-ACPs. For synthesis of saturated fatty acids, the elongation process is repeated until the acyl chain reaches the desired length (usually 14–18 carbons in Gram-negative bacteria). In the α and γ

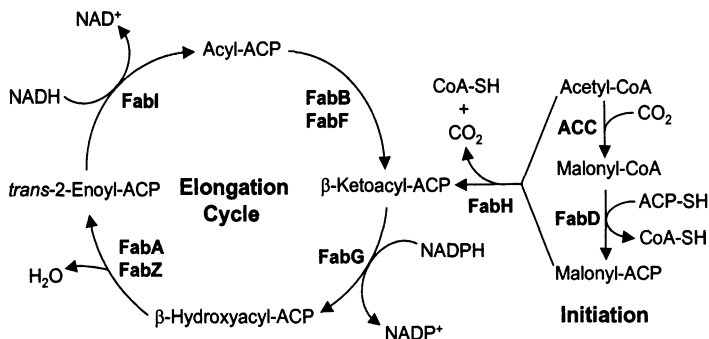


Figure 1. Pathway of bacterial fatty acid biosynthesis. Acetyl-CoA is carboxylated by the AccABCD acetyl-CoA carboxylase complex (ACC) to form malonyl-CoA, which is then transferred to holo-acyl carrier protein (ACP-SH) by malonyl-CoA:ACP transacylase (FabD). FAS is then performed in a repeated cycle of condensation by various β -ketoacyl-ACP synthases (FabB, FabF or FabH), reduction by β -ketoacyl-ACP reductase (FabG), dehydration by β -ketoacyl-ACP dehydrases (FabA or FabZ) and reduction by enoyl-ACP reductases (FabI; or FabK or FabL in some bacteria; *P. aeruginosa* uses FabI and FabK). FabH is thought to only catalyze the initial condensation of acetyl-CoA and malonyl-CoA, whereas in subsequent cycles FabB and FabF catalyze condensation of acyl-ACP and malonyl-ACP, releasing holo-ACP and CO_2 . All of the enzymes, with the exception of FabH, were shown to exist and function in *P. aeruginosa*. Other abbreviation: CoA-SH, reduced coenzyme A.

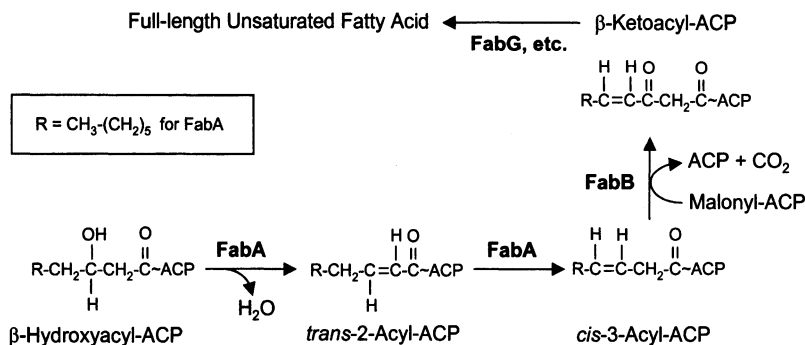


Figure 2. The anaerobic pathway for synthesis of unsaturated fatty acids. This pathway is found in the α and γ Proteobacteria. In the reactions shown, FabA catalyzes the isomerization of the *trans*-2 double bond to a *cis*-3 double bond at the 10 carbon stage of FAS. The *cis*-3 product is then condensed with malonyl-ACP to form a β -ketoacyl-ACP, essentially bypassing the enoyl-ACP reductase (FabI) step and thus maintaining the double bond. In subsequent rounds of elongation, the acyl chain is then elongated to the full-length unsaturated fatty acid.

Proteobacteria, saturated and unsaturated FAS are linked via the anaerobic unsaturated fatty acid biosynthetic pathway (Figure 2).

The unsaturated fatty acids are produced via an isomerization of the *trans*-2 double bond to a *cis*-3 double bond at the 10 carbon stage of

synthesis^{16, 35}. This isomerization is catalyzed by FabA. The *cis*-3 product is then condensed with malonyl-ACP to form a β -ketoacyl-ACP, essentially bypassing the enoyl-ACP reductase (FabI) step and thus maintaining the double bond. In subsequent rounds of elongation, the acyl chain is then extended to the full-length product. Since both FabA and FabB play essential roles in this process, mutations in either locus lead to unsaturated fatty acid auxotrophy⁴². FabF is responsible for modulation of the chain length of unsaturated fatty acids: At low temperature, *P. aeruginosa* makes more *cis*-vaccenic acid, and this adaptation is impaired in *fabF* mutants⁵⁶.

2.1. Acyl Carrier Protein

In most bacteria, the growing acyl chain remains tethered to ACP during *de novo* fatty acid biosynthesis. One exception is the FAS I system of mycobacteria in which growing acyl chains are tethered to coenzyme A⁴. Bacterial ACPs are small, highly acidic proteins: for example, the *P. aeruginosa* ACP is 8,583 Da and has a calculated pI of 3.8⁵⁶. To gain functionality, the apo-ACP encoded by the chromosomal *acpP* requires covalent modification by transfer of the 4'-phosphopantetheine group from coenzyme A to a conserved serine residue (Figure 3). In *P. aeruginosa*, this reaction is catalyzed by a new type of phosphopantetheinyl transferase, PcpS, which functions in both fatty acid and siderophore synthesis²⁵. Although PcpS is peripheral to the

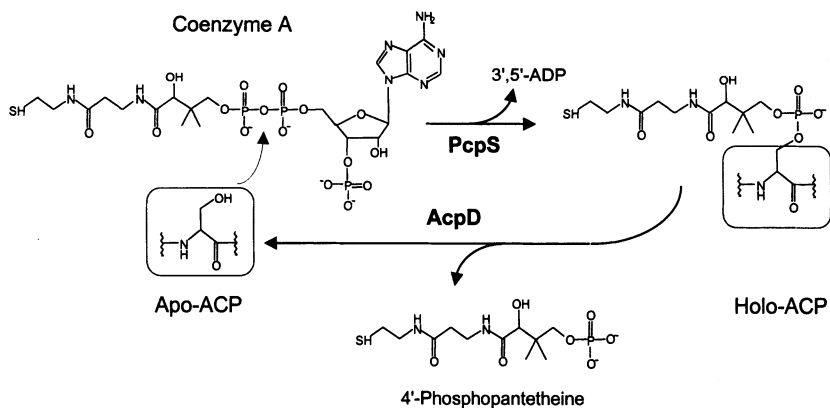


Figure 3. Reactions involved in the modification of ACP. Apo-ACP is covalently modified by holo-ACP synthase (PcpS in *P. aeruginosa*) by transfer of the 4'-phosphopantetheine prosthetic group from coenzyme A to a conserved serine in apo-ACP resulting in formation of holo-ACP. This reaction can be reversed by holo-ACP phosphodiesterase (AcpD), although the physiological role of this reaction remains obscure. The stippled arrow indicates the site of ester bond formation between the serine hydroxyl and the 4'-phosphopantetheine of coenzyme A.

Fab pathway, it is nonetheless an essential component of this pathway, which explains its essentiality for bacterial growth²⁵ (N. Barekzi and H.P. Schweizer, unpublished observations). Although its physiological role remains obscure, in *Escherichia coli*, an ACP phosphodiesterase (AcpD) was proposed to convert holo-ACP to apo-ACP by removal of the 4'-phosphopantetheine prosthetic group²⁶ (Figure 3) but the gene encoding this enzymatic activity remains yet to be identified. The relationship of this enzyme to FAS, if any, remains unknown. The *acpP* gene is located in the major *P. aeruginosa* *fab* gene cluster that also contains *fabD*, *fabG* and *fabF1*⁵⁶. The *P. aeruginosa* ACP has been purified and its function demonstrated in vitro^{43, 56}. Besides *acpP*, the *P. aeruginosa* chromosome contains two additional genes for probable ACPs (Table 1). However, although one of them may be clustered with a potential Fab enzyme, FabH or β -ketoacyl-ACP synthase III, neither of these probable ACPs can apparently substitute for the *acpP*-encoded ACP in FAS since *acpP* is an essential gene⁵⁶.

2.2. Acetyl-CoA Carboxylase

Fatty acid synthesis is initiated by carboxylation of acetyl-CoA. This ATP-dependent reaction is catalyzed by the multisubunit acetyl-CoA carboxylase complex (AccABCD). Since *P. aeruginosa* contains homologs of all four enzyme subunits, whose function was demonstrated in *E. coli*, it can be assumed that the overall reaction proceeds in a very similar fashion in the two bacteria⁵. The acetyl carboxylase reaction can be divided into two half reactions. In the first reaction, the CO₂ moiety of bicarbonate is activated and transferred to biotin carboxylase carrier protein (BCCP; AccB) in a reaction catalyzed by biotin carboxylase (AccC). In the second reaction, the CO₂ group is transferred from carboxybiotin to acetyl-CoA to form malonyl-CoA in a reaction catalyzed by the two carboxyl transferase subunits (AccA and AccD). The biotin cofactor must be covalently attached to the AccB subunit via a biotin protein ligase, another peripheral, albeit essential, FAS-related protein. A gene, PA4280, that is homologs to the bifunctional biotin protein ligase/biotin repressor gene *birA* of *E. coli* is present in *P. aeruginosa*, although its functionality has not yet been demonstrated.

2.3. Malonyl-CoA:ACP Transacylase

Malonyl-CoA:ACP transacylase is encoded by the *fabD* gene, which is located in the major *P. aeruginosa* *fab* gene cluster that also contains *acpP*, *fabF1* and *fabG*⁵⁶. This enzyme catalyzes the transthioesterification of malonate from coenzyme A to ACP. A temperature-sensitive *fabD* allele blocks *P. aeruginosa* growth at nonpermissive temperatures indicating that malonyl-CoA:ACP transacylase is not only essential for FAS but also that *fabD* is the

only *P. aeruginosa* gene encoding this Fab activity⁵⁶. The *P. aeruginosa* FabD protein has been purified and exhibits malonyl-CoA:ACP transacylase activity in vitro^{43, 56}.

2.4. β -Ketoacyl-ACP Synthases

The *P. aeruginosa* genome contains genes for several potential β -ketoacyl-ACP synthases (KAS) or condensing enzymes: *fabB* encoding KAS I, *fabF1* encoding KAS II, *fabF2* encoding another probable KAS II enzyme and *fabH2*, which encodes a KAS III candidate enzyme. All of these are located in distinct chromosomal locations.

The *fabB* gene is the second gene of the *fabAB* operon⁴². Mutants defective in *fabB* are unsaturated fatty acid auxotrophs, consistent with a major role of this enzyme in unsaturated FAS⁴². FabB was essential for chain elongation in an in vitro FAS system, indicating that it can also perform all elongation steps in saturated FAS⁴³.

The *fabF1* gene is located in the major *P. aeruginosa* *fab* gene cluster that also contains *acpP*, *fabD* and *fabG*⁵⁶. A *fabF1* insertion mutant was generated and contained substantially reduced levels of *cis*-vaccenic acid, consistent with a crucial role of FabF1 in unsaturated FAS⁵⁶. Thus, both FabB and FabF1 play important roles in unsaturated FAS. In *E. coli*, FabB is required for elongation of an intermediate early precursor in unsaturated FAS, whereas FabF is required for the last step in the unsaturated pathway^{8, 16}, and in *P. aeruginosa* FabB and FabF1 probably play similar roles. Since a *fabB* mutant is seemingly unimpaired in saturated FAS, it appears that, like FabB, FabF seems to be able to perform all elongation steps in this pathway.

In *E. coli*, FabH (or KAS III) is apparently responsible for the synthesis of short-chain acyl-ACPs. This synthase is distinct from synthases I and II because of its use of acetyl-CoA rather than acetyl-ACP as the immediate precursor of the methyl end of the nascent fatty acid^{8, 16}. Since *E. coli* FabH produces mainly four carbon keto acids and cannot make long-chain fatty acids, KAS III was attributed a role in the initiation of FAS. This remains unproven, however, because *fabH* mutants have not been reported. Although *P. aeruginosa* contains at least one potential gene for a KAS III enzyme, *fabH2*, purified FabH was not required for FAS from acetyl-CoA and malonyl-CoA in an in vitro FAS system⁴³. Albeit not yet proven, FabB may be able to decarboxylate malonyl-ACP to acetyl-ACP and then condense these two molecules to initiate the Fab cycle¹⁶. Even though *fabH2* maps next to another probable ACP-encoding gene, this does not necessarily imply a role in FAS but rather could also reflect its role in another ACP- and fatty acid modification-requiring pathway. This notion is supported by the fact that this probable ACP cannot substitute for the *acpP*-encoded ACP of the Fab pathway.

2.5. β -Ketoacyl-ACP Reductase

As was demonstrated for other bacteria, the *P. aeruginosa* β -ketoacyl-ACP reductase is encoded by *fabG*, an essential gene shown by characterization of a temperature-sensitive mutant⁴³. The *fabG* gene is located in the major *P. aeruginosa* *fab* gene cluster that also contains *acpP*, *fabD* and *fabF1*⁵⁶. The *P. aeruginosa* FabG protein was purified and its NADPH-dependent β -ketoacyl-ACP reductase activity was essential for FAS from metabolic precursors in an in vitro system⁴³.

2.6. β -Hydroxyacyl-ACP Dehydrases

The dehydration of β -hydroxyacyl-ACPs in *P. aeruginosa* is catalyzed by two enzymes, FabA and FabZ, two activities that were previously characterized in *E. coli*³².

The *P. aeruginosa* *fabA* gene is the first gene in the *fabAB* operon and non-polar *fabA* insertion mutants are unsaturated fatty acid auxotrophs⁴². This phenotype is consistent with FabA dehydrating β -hydroxyacyl-ACPs and then isomerizing the resulting *trans*-2-acyl-ACPs to *cis*-3-acyl-ACPs, the double bonds of which become the *cis* double bond of unsaturated fatty acids after several cycles of chain elongation (Figure 2)¹⁶. Since overproduction of FabA in *E. coli* increases saturated FAS, the FabA-produced *trans*-enoyl product is also capable of entering the saturated FAS pathway¹⁵.

The finding that saturated FAS was unaffected in a *fabA* mutant strongly argued for the presence of a second dehydrase, FabZ, which was subsequently cloned, overexpressed and purified⁴³. As in other Gram-negative bacteria, *P. aeruginosa* *fabZ* is found within a cluster of lipid A biosynthetic genes. Unlike FabA, purified *P. aeruginosa* FabZ was required for in vitro synthesis of fatty acids from acetyl-CoA and malonyl-CoA indicating that it functions on all chain lengths encountered during fatty acid biosynthesis⁴³.

2.7. Enoyl-ACP Reductase

Enoyl-ACP reductase catalyzes the last step in each elongation cycle. Three distinct types of bacterial enoyl-ACP reductases exist (FabI, FabL and FabK)³³. Mutant analysis revealed that *P. aeruginosa* contains at least two types of enoyl-ACP reductases, the triclosan-sensitive FabI-mediated enoyl-ACP reductase activity and a triclosan-insensitive enoyl-ACP reductase activity⁴¹, which was subsequently termed FabK³³. Whereas it has been shown that the *P. aeruginosa* *fabI* gene is the last gene in an oligopeptide permease operon (J. Cusick and H.P. Schweizer, unpublished observations), the *fabK* gene and its product remain unidentified. *P. aeruginosa* FabI was purified and

Table 2. Fatty acid biosynthetic genes in the genomes of *P. putida* KT2440 and *P. syringae* DC3000.

Gene	<i>P. putida</i>			<i>P. syringae</i>		
	Annotation	Nucleotide (% identity) ¹	Protein (% identity) ¹	Annotation	Nucleotide (% identity) ¹	Protein (% identity) ¹
<i>fabA</i>	PP4174	85	89	PSPT02211	87	92
<i>fabB</i>	PP4175	84	83	PSPT02210	82	82
<i>fabD</i>	PP1913	74	63	PSPT03833	75	67
<i>fabF</i>	PP1916	80	80	PSPT03830	78	79
<i>fabG</i>	PP1914	78	83	PSPT03832	75	82
<i>fabH</i>	PP4379	ND	26	PSPT01916	ND	30
	PP4545	ND	26	PSPT01948	ND	29
				PSPT04094	ND	25
<i>fabI</i>	ND ^{2,3}	ND	ND	PSPT03721	83	90
<i>fabZ</i>	PP1602	83	86	PSPT01545	81	86
<i>acpP</i>	PP1915	84	89	PSPT03831	85	89

¹The respective *P. aeruginosa* *fab* genes and translated coding sequences (see Table 1 for annotations and enzyme functions) were used in blastn and blastp searches of the Comprehensive Microbial Resource (CMR) databases at The Institute for Genomic Research (<http://tigrblast.tigr.org/cmr-blast/>), and the percent identity of the listed genes and proteins to the respective *P. aeruginosa* homologs was determined. Annotations are those provided by the CMR.

²ND, not detectable.

³A blastp search using the *P. aeruginosa* FabI amino acid sequence revealed several potential reading frames belonging to the oxidoreductase and short chain dehydrogenase/reductase families.

its NADH-dependent enoyl-ACP reductase activity was demonstrated in vitro^{40, 41, 43}. Although there is a high level of genome conservation between *P. aeruginosa* and *P. putida*, it is interesting to note that *fabI* is apparently absent from the *P. putida* KT2440 genome⁷⁰ (Table 2).

3. FATTY ACID BIOSYNTHETIC ENZYMES IN OTHER PSEUDOMONADS

The recent completion of the *P. putida* KT2440⁷⁰ and *P. syringae* (www.tigr.org) genomes allows a closer examination of the presence, genomic organization and degree of conservation of the *fab* genes and their products (Table 2).

With the exception of *fabH* and *fabI*, the degree of conservation, in terms of *fab* (and *acpP*) gene (product) similarity and organization, in the three genomes is striking: (a) they all contain a major *fab* gene cluster and within this cluster the gene order is the same (*fabF-acpP-fabG-fabD*), with *fabH* being absent from this cluster; (b) all three genomes contain a probable *fabAB* operon (this has been proved for *P. aeruginosa*); (c) the *fabZ* gene is located

outside of the *fab* gene clusters; (d) the degrees of similarity of the genes and their products are very high, both at the nucleotide and amino acid level; and (e) all genomes encode at least one additional probable β -ketoacyl-ACP synthase II (FabF2). However, some noteworthy differences do exist. First, although all genomes contain putative FabH homologs, the degree of conservation is low and therefore a role of this gene and its product in fatty acid biosynthesis becomes even more questionable. Second, although there is a high level of genome conservation between *P. aeruginosa* and *P. putida* (85% of the coding regions are shared), the *fabI* gene encoding enoyl-ACP reductase is seemingly missing from the *P. putida* KT2440 genome. Therefore, *P. putida* most likely contains a FabK- or FabL-type enoyl-ACP reductase.

Given the presence of almost identical *fab* and *acpP* gene complements in *P. aeruginosa*, *P. putida* and *Pseudomonas syringae*, the fatty acid biosynthetic pathways are probably very similar in these three pseudomonads.

4. POSTSYNTHETIC MODIFICATIONS

Bacterial lipids and fatty acids undergo various postsynthetic modifications. Two of the most significant modifications known are *cis-trans* isomerization and formation of cyclopropane fatty acids. Both these modifications do not involve free fatty acids, or coenzyme A- and ACP-linked fatty acids, but rather use mature phospholipid molecules that are already incorporated into and functioning within membrane bilayers.

4.1. *Cis-Trans* Isomerization

The *cis-trans* isomerization of fatty acids plays an important role in solvent tolerance of bacteria^{86, 87}. Although among pseudomonads this postsynthetic modification has been best characterized in *P. putida*^{51, 59}, it occurs in many other bacteria⁵⁴. Since the *cis* unsaturated fatty acid isomers are those synthesized by the cell, they are present in many bacteria but the *trans* isomers are less widespread⁵⁴. It has been proposed that *cis-trans* isomerization of unsaturated fatty acids may be a possible control mechanism of membrane fluidity by increasing membrane rigidity^{59, 87}. In response to exposure to various solvents and aromatic compounds, various *P. putida* strains rapidly shift their *cis* to *trans* ratio, and this shift does not require *de novo* protein synthesis. The (mainly) palmitoleic (C16) and vaccenic acid (C18) *trans* isomers are directly and rapidly (within 1 min of exposure) synthesized from the respective *cis* isomers without shift in the position of the double bond^{38, 59, 87}. It is currently unclear how cells sense the presence of organic solvents and activate *cis-trans* isomerase (Cti) enzymes. Genes encoding Cti enzymes have been cloned from

various *P. putida* strains^{46, 51} and also from *Pseudomonas oleovorans*⁸¹. These studies indicated that Cti is a periplasmic protein. A *P. putida* Cti null mutant was unable to isomerize *cis*-unsaturated fatty acids and it was used to demonstrate that *cis*–*trans* isomerization of fatty acids was required to improve the survival of this strain to toluene and high temperatures⁵¹. Since *cis*–*trans* isomerization occurs rapidly and only partially confers solvent tolerance, it is considered a short-term response mechanism and other, longer-term mechanisms, such as phospholipid head group modifications and active efflux mechanisms clearly contribute to the overall solvent tolerance of many bacteria⁸⁶. Although efflux has been shown to also play an important role of *P. aeruginosa* organic solvent tolerance⁵⁸, it is not known whether a putative Cti enzyme (Table 1) participates in this process.

4.2. Cyclopropane Fatty Acid Synthesis

Cyclopropane fatty acids (CFAs) are widely distributed among the bacteria and are formed by the addition of a methyl group from *S*-adenosylmethionine across the double bond of unsaturated fatty acids²⁸. CFAs are usually synthesized at the onset of stationary phase. Despite the fact that the unsaturated double bond in membrane phospholipids is normally sequestered deep within the hydrophobic interior of the lipid bilayer, this reaction is catalyzed by a soluble CFA synthase in *E. coli*¹⁰². *E. coli* CFA synthase expression is inducible by virtue of the stationary-phase sigma factor RpoS¹⁰¹ and enzyme activity is transient due to RpoH-dependent proteolysis¹¹. Among the pseudomonads, induced synthesis of CFA synthase activity was reported in *Pseudomonas denitrificans*^{49, 50}, *Pseudomonas fluorescens*¹⁷ and *Pseudomonas aeruginosa*²¹. Although the expression of *P. denitrificans*⁵⁰ and *P. aeruginosa*²¹ CFA synthases seemed to be growth-phase dependent, the molecular mechanisms governing this expression pattern remain unknown. In some pseudomonads, CFA can make up a large proportion of cellular fatty acids. For example, in *P. putida*, DOT-T1 cells grown in Luria–Bertani medium *cis*-9,10-methylene hexadecanoic acid (C17:cyclopropane) represented up to 30% of the total fatty acid content⁸⁷. Although its biological significance remains obscure, the accumulation of C17:0 CFA was cited as a conserved trait in fluorescent pseudomonads with antifungal activity²².

5. OTHER ENZYMES RELATED TO FATTY ACID SYNTHESIS

The *P. aeruginosa* genome contains several other genes whose products could potentially be related to or function in fatty acid biosynthesis and/or

modification (Table 1). For example, although it is generally believed that in γ Proteobacteria unsaturated fatty acids are only produced via the classic anaerobic pathway, which requires the key enzyme FabA, *P. aeruginosa* also encodes a potential fatty acid desaturase. Desaturases are believed to function in the only other known pathway of unsaturated FAS where they catalyze desaturation of full-length fatty acids to unsaturated derivatives. However, desaturases are thought to occur only in aerobic organisms such as *Bacillus subtilis*¹.

6. BIOLOGICALLY SIGNIFICANT ACYL TRANSFER REACTIONS

Despite the fact that pseudomonads require fatty acids for biosynthesis of various constituent cellular macromolecules of biological importance, including phospholipids, rhamnolipids, lipid A, lipoproteins, etc., many of the pathways involved in the biosynthesis of these molecules have not been elucidated in any great detail in these bacteria. Although the available genome sequences reveal many conserved genes that allow an elucidation of conserved themes among related pathways of Gram-negative organisms, the exact function and architecture of many of these pathways awaits genetic and biochemical analyses. Nonetheless, several acyl transfer-requiring pathways in pseudomonads have been characterized in greater detail and will be summarized here (other chapters in these volumes will present more details on some of these topics, particularly quorum sensing, lipopolysaccharide and polyhydroxyalkanoic acids).

6.1. Acylated Homoserine Lactone Synthesis

Acylated homoserine lactones (AHLs) are involved in cell-cell communication of Gram-negative bacteria⁶³. Many pseudomonads produce AHLs, including *Pseudomonas aeruginosa*^{77, 79, 80}, *Pseudomonas fluorescens*⁵⁷, *Pseudomonas putida*⁹⁷, *Pseudomonas chlororaphis*¹³, *Pseudomonas aureofaciens*¹⁰⁶ and many other plant-associated pseudomonads¹⁰.

Among pseudomonads, quorum-sensing is best understood in the important opportunistic pathogen *P. aeruginosa*, where it is required for expression of numerous virulence factors^{18, 63, 95}. *P. aeruginosa* produces two LuxI-type AHL synthases, LasI and RhII, which are required for synthesis of butyryl-HSL and 3-oxo-dodecanoyl-HSL, respectively. It has been well established that the acyl group is derived from acyl-ACPs from the fatty acid biosynthetic pathway and that the HSL moiety is derived from *S*-adenosylmethionine^{40, 41, 43, 67, 76, 100}. A reaction mechanism was proposed for AHL synthesis by LuxI-type proteins^{63, 103} (Figure 4). Most of the different acyl chains found in the AHLs,

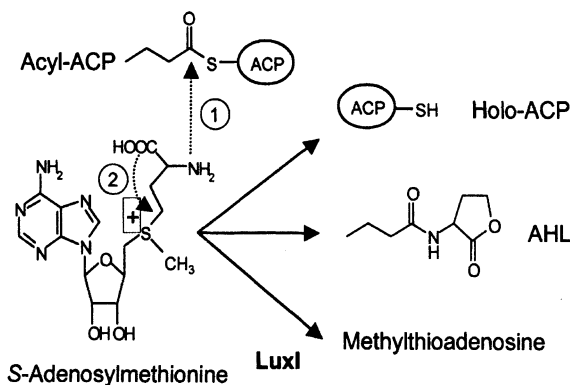


Figure 4. Acyl transfer in synthesis of AHL. LuxI enzymes use *S*-adenosylmethionine (SAM) and specific acyl-ACP as substrates for synthesis of AHL. The enzyme directs formation of an amide linkage between SAM and the acyl moiety of the acyl-ACP via the amino group nucleophile of SAM (stippled arrow labeled 1). Subsequent lactonization (stippled arrow labeled 2) of the ligated intermediate with the concomitant release of methylthioadenosine and holo-ACP occurs, resulting in the formation of AHL. Shown in the figure is *N*-butyryl-homoserine lactone, which is synthesized by RhlI of *P. aeruginosa*.

namely 3-unsubstituted, 3-hydroxy and 3-oxo, can directly be derived from the Fab pathway (Figure 1) by competing with the respective Fab enzymes for the required acyl-ACP substrates. For example, *P. aeruginosa* LasI competes with FabG for its 3-oxo-acyl-ACP substrates and FabG activity seems to be a determining factor of acyl chain lengths found in 3-oxo-AHLs⁴³.

6.2. Rhamnolipid Synthesis

Rhamnolipids are tenso-active glycolipids containing one (mono-rhamnolipid) or two (di-rhamnolipid) L-rhamnose molecules⁷². These compounds are biodegradable and have potential industrial and environmental applications^{39, 64}, as well may serve as possible biocontrol agents against plant zoosporic pathogens⁹⁶.

In *P. aeruginosa* it has been shown that the metabolic precursors required for rhamnolipid synthesis are derived from the Fab pathway via the presumably NADPH-dependent β -ketoacyl-ACP reductase, RhlG, which is specifically involved in rhamnolipid synthesis⁹ (Figure 5). This enzyme reduces β -ketodecanoyl-ACP from the Fab pathway to β -hydroxydecanoyl-ACP. The β -hydroxydecanoyl moiety is then transferred to coenzyme A by an unknown β -hydroxydecanoyl-ACP:CoA transacylase (PhaG?) with concomitant release of holo-ACP. Next, β -hydroxydecanoyl- β -hydroxydecanoate is formed by combining two β -hydroxydecanoyl-CoA molecules in the polyhydroxyalkanoate synthase (PhaC) catalyzed reaction. *P. aeruginosa*

pseudomonads, including *P. aeruginosa* and *P. putida*, are able to synthesize and accumulate large amounts of PHAs consisting of various β -hydroxy fatty acids. These PHAs are of medium chain length (PHA_{MCL}), ranging from 6 to 14 carbons^{2, 61}. The composition of PHAs depends on the PHA synthases, the carbon source and the metabolic routes involved in their synthesis^{89, 90}. In *P. putida* KT2442 cells using fatty acids as carbon source, β -oxidation is the main pathway⁴⁷. In contrast, when cells grow on carbon sources that are metabolized to acetyl-CoA, for example, gluconate or acetate, fatty acid biosynthesis is the main route of synthesis (Figure 5). In some instances, for example, during growth on hexanoate, both metabolic routes function simultaneously⁴⁷. Evidence was also presented that during growth of *P. putida* KT2442 on medium-chain-length fatty acids, PHA precursors can be generated by elongation of these fatty acids with an acetyl-CoA molecule⁴⁷.

Although it has been established that the FabG-mediated generation of β -hydroxyacyl-ACPs is a required step in PHA_{MCL} synthesis⁹¹, purified *P. aeruginosa* PHA_{MCL} synthases (PhaC) use β -hydroxydecanoyl-CoA as the substrate⁸³. The β -hydroxyacyl-ACPs, which are the corresponding intermediates of the Fab pathway, must therefore be converted to the corresponding β -hydroxyacyl-CoA derivatives. This direct link between fatty acid biosynthesis and PHA synthesis is provided by a transacylase enzyme (PhaG), which transfers the β -hydroxyacyl moiety from the ACP thioester to coenzyme A⁸⁸. The PhaG transacylase-mediated pathway seems widespread among pseudomonads^{44, 45, 88}. The medium-chain length PHAs are then synthesized by polymerization of β -hydroxyacids by PHA synthase (PhaC)^{83, 99}. Although β -hydroxydecanoic acids are constituents of both *P. aeruginosa* PHAs and rhamnolipids, PHA synthesis during hexadecane fermentation was found to occur only during active cell growth, while significant rhamnolipid synthesis was observed only at the onset of and during stationary phase growth¹². The latter observation is consistent with previous findings which showed that the expression of both rhamnosyltransferases is governed by the quorum-sensing regulatory circuit^{73, 85}.

6.4. Lipid A Synthesis

Lipopolysaccharide of *P. aeruginosa* is an important virulence factor⁹². Like other LPS molecules, it contains a hydrophobic domain known as lipid A (or endotoxin), a nonrepeating “core” oligosaccharide and a distal polysaccharide (or O-antigen).

The lipid A of *P. aeruginosa* LPS contains six acyl moieties⁸⁴ (Figure 6). During lipid A biosynthesis these are sequentially incorporated using the respective acyl-ACP substrates derived from the Fab pathway⁸⁴. The first 10 carbon fatty acid is incorporated by a UDP-*N*-acetylglucosamine acyltransferase

(LpxA). This enzyme has an absolute requirement for an ACP thioester and is selective for β -hydroxydecanoyl-ACP²⁰. Single amino acid substitutions can convert *P. aeruginosa* LpxA to a 14-carbon-specific acyltransferase that has the same selectivity for β -hydroxymyristoyl as the *E. coli* LpxA¹⁰⁷. The second fatty acid incorporated is the 12 carbon β -hydroxylaurate moiety from β -hydroxylauoyl-ACP by LpxD to generate UDP-2,3-diacylglucosamine. After formation of the disaccharide, the final two secondary fatty acids that are incorporated per monosaccharide are laurates (C12)^{66, 84}. These reactions are catalyzed by two enzymes that are orthologs of *E. coli* LpxL.

Pathogenic bacteria synthesize different forms of lipid A in response to environmental conditions that include limiting Mg^{2+} and conditions encountered during mammalian infections⁸⁴. One such modification of LPS found in *P. aeruginosa* isolated from cystic fibrosis airways is the addition of a secondary palmitoyl chain²⁴ (Figure 6). In contrast to all other acyl residues found in lipid A, including the secondary laurate residues, the secondary palmitoyl chain is not incorporated by an ACP-dependent mechanism⁸⁴.

6.5. Phospholipid Synthesis

Phospholipids are essential for the integrity and biological functions of bacterial membranes¹⁶, and phospholipid modifications are involved in biological adaptations of bacteria, for example, solvent tolerance^{82, 86, 87}. Although most pseudomonads contain many of the phospholipid species found in *E. coli*, for example, phosphatidylethanolamine, they also have the capability to biosynthesize some phospholipids that are not found in *E. coli* (e.g., ornithine-containing lipids in several pseudomonads⁵² and phosphatidylcholine in *P. aeruginosa*¹⁰⁴).

In Eubacteria, phospholipids are comprised of an *sn*-glycerol-3-phosphate (G3P) molecule, whose glycerol backbone is esterified with two fatty acids¹⁶. The fatty acid substrates are acyl-ACPs from the fatty acid biosynthetic pathway. The fatty acid moieties are transferred to G3P in two successive reactions. The first enzyme transfers fatty acids to the 1 position of the glycerol backbone and the second enzyme then esterifies the 2 position with a second fatty acid. The asymmetrical distribution of fatty acids between the 1 and 2 positions is controlled in part by the acyl chain specificity of the acyltransferases. The molecule resulting from diacylation of G3P is phosphatidic acid. Phosphatidic acid is an intermediate in phospholipid synthesis and is rapidly converted to CDP-diacylglycerol by CDP-diacylglycerol synthase. Other lipids are derived from CDP-diacylglycerol by the action of various modifying enzymes. For example, phosphatidylserine is derived from CDP-diacylglycerol by condensation with serine, a reaction catalyzed by phosphatidylserine synthase. Phosphatidylserine is then decarboxylated by

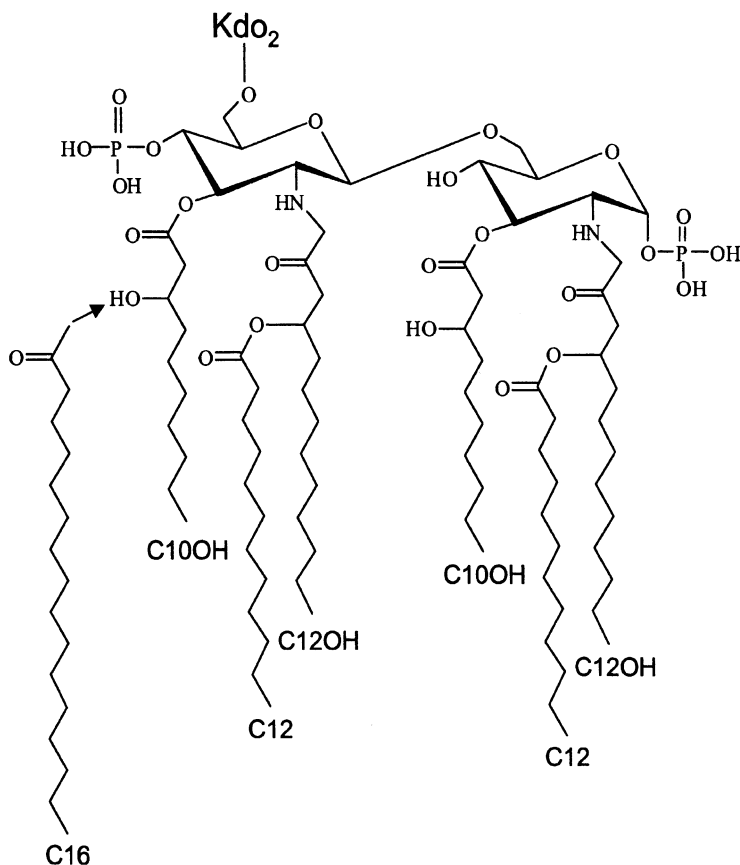


Figure 6. Structure of *P. aeruginosa* lipid A. The hexacyl disaccharide-1,4'-bis-Phosphate unit of lipid A contains two 3-deoxy-D-manno-octulosante (Kdo) units that are indicated by Kdo₂. Other abbreviations: C10OH, β-hydroxydecanoate; C12OH, β-hydroxylaurate; C12, laurate; C16, palmitate. The palmitate modification is only found in certain clinical isolates and the site of modification is indicated by the arrow. With the exception of the C16 palmitoyl chain, all other fatty acids are incorporated by enzymes that require ACP-linked acyl moieties.

phosphatidylserine decarboxylase to form phosphatidylethanolamine. Even though little is known about the phospholipid biosynthetic pathways in pseudomonads, it can be assumed that the basic pathways in these bacteria are very similar to the one characterized in *E. coli* because the genomes of *P. aeruginosa* and other pseudomonads encode homologs of the *E. coli* phospholipid biosynthetic enzymes. However, the presence of unique phospholipids in the pseudomonads necessitates unique enzymes. One of these was recently demonstrated in *P. aeruginosa*, which synthesizes phosphatidylcholine via the choline-dependent pathway¹⁰⁴. In this pathway,

phosphatidylcholine synthase (Pcs) condenses choline directly with CDP-diacylglycerol to form phosphatidylcholine in one step^{60, 104}.

7. FATTY ACID BIOSYNTHESIS AS TARGET FOR ANTIBACTERIAL DRUGS

Due to the essential nature of many of the enzymes involved in bacterial fatty acid biosynthesis, the Fab pathway offers some attractive targets for antibacterial drugs. Although a good number of FAS inhibitors have been identified (Figure 7), many of these drugs target the same Fab enzymes^{8, 35}, and only the antimycobacterial drugs isoniazid and ethionamide are currently in clinical use^{4, 94, 105}. Whereas no inhibitors of acetyl-CoA carboxylase (ACC), malonyl-CoA:ACP transacylase (FabD) and β -ketoacyl-ACP reductase (FabG) are known, an inhibitor of β -hydroxyacyl-ACP dehydrase (FabA), and several inhibitors of β -ketoacyl-ACP synthases (FabB, FabF and FabH), as well as enoyl-ACP reductase (FabI) have been identified and characterized.

7.1. Dehydrase Inhibitors

The only known inhibitor of β -hydroxyacyl-ACP dehydrase activity is the specific-mechanism-activated FabA inhibitor *cis*-3-decynoyl-*N*-acetylcysteamine⁷. It is an analogue of the *cis*-3-decenoyl-ACP normally

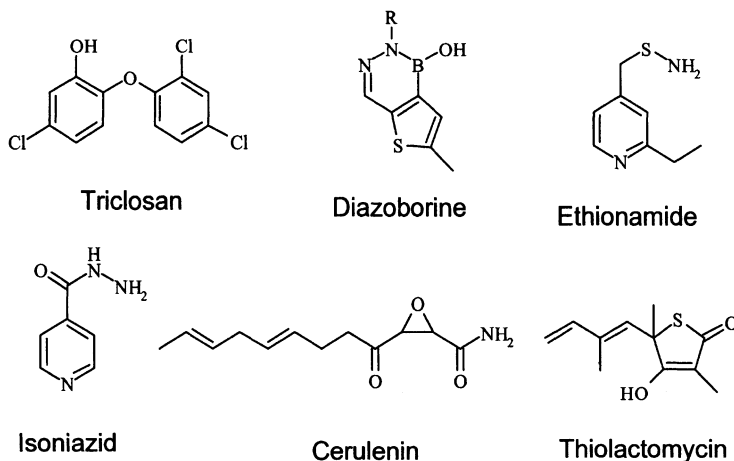


Figure 7. Inhibitors of FAS. The R group of diazaborine indicates the position of the variable group.

produced by the isomerase activity of FabA³⁵. The susceptibility of pseudomonads to this compound has not yet been reported.

7.2. Synthase Inhibitors

β -Ketoacyl-ACP synthase (KAS) inhibitors include cerulenin and thiolactomycin. Cerulenin was originally isolated from culture filtrates of *Cephalosporium caerulens* as an antifungal antibiotic with broad antimicrobial spectrum^{29, 75}. Cerulenin is a potent inhibitor of both type I and type II fatty acid synthases. Type II KAS enzymes are commonly separated into two classes based on their cerulenin sensitivity. Whereas *E. coli* KAS I (FabB) and KAS II (FabF) are efficiently inhibited by cerulenin, KAS III (FabH) is not significantly affected by this antibiotic. KAS I and KAS II are irreversibly inhibited by cerulenin and the mechanism of inhibition involves covalent cross-linking of the KAS active site cysteine to cerulenin⁸.

Thiolactomycin was originally isolated from a *Nocardia* strain^{65, 74}. The compound lacked activity against type I FAS enzymes but was very active against various bacterial species. Thiolactomycin was shown to be a specific inhibitor of FAS^{30, 31} and inhibits all three known KAS enzymes of *E. coli*⁸, as well as the mycobacterial KasA and KasB KAS enzymes⁵⁵. Thiolactomycin acts as a competitive inhibitor with malonyl-ACP⁷¹. *P. aeruginosa* wild-type strains are resistant to cerulenin and thiolactomycin because both of these antibiotics are efflux pump substrates⁹³.

7.3. Enoyl-ACP Reductase Inhibitors

The enoyl-ACP reductase FabI is the target of at least three classes of antimicrobial compounds, including triclosan, diazaborine derivatives, and isoniazid and ethionamide.

Triclosan is a trichlorinated biphenyl ether and is the active ingredient in many consumer products that exhibit “antibacterial” properties⁶. It was shown to specifically target FabI^{34, 36, 62}, thus inhibiting the enoyl-ACP reductase step of the bacterial Fab pathway. The sensitivity of enoyl-ACP reductases seems to be confined to enzymes that use NADH or NADPH. The other two types of enoyl-ACP reductases found in bacteria, FabK and FabL, are triclosan-insensitive enzymes³³.

Diazaborine derivatives inhibit FabI activity by formation of a covalent linkage between the boron atom of the inhibitor and the ribose moiety of the NAD cofactor³.

The enoyl-ACP reductase inhibitors isoniazid and ethionamide have a very narrow range of efficacy. They are specific inhibitors of mycolic acid synthesis and it is therefore not surprising that they both are very effective antimycobacterial drugs^{4, 8, 35}.

Pseudomonas aeruginosa is resistant to very high levels (>1 mg/ml) of triclosan because this compound is an excellent efflux pump substrate¹⁴. Furthermore, *P. aeruginosa* contains the triclosan-insensitive enoyl-ACP reductase FabK, which may further contribute to intrinsic triclosan resistance^{33, 35, 41}. The sensitivity of diazaborines against pseudomonads is unknown, and both isoniazid and ethionamide were found to be ineffective against *P. aeruginosa* (H.P. Schweizer, unpublished observations). More recently, the discovery of a novel class of FabI-directed antibacterial agents was reported but their efficacy against *P. aeruginosa* was marginal⁷⁸.

8. CONCLUSIONS

Although the research conducted over the last few years allowed an elucidation of the molecular mechanisms of fatty acid biosynthesis and its related pathways mostly in *P. aeruginosa* and a few other pseudomonads, much work remains to be done.

Despite the availability of genome sequences and bioinformatic analyses for several pseudomonads, the function of many of the *P. aeruginosa* genes listed in Table 1 remains unproven or unknown. Although *P. aeruginosa* was the first Gram-negative bacterium for which two enoyl-ACP reductases were predicted, FabI and FabK, repeated attempts aimed at identifying the *fabK* structural gene have met with failure by different research groups. With *fabI* apparently absent from the *P. putida* genome, the identification of its enoyl-ACP reductase, probably a FabK homolog, remains to be accomplished. Since none of the three *Pseudomonas* genome sequences encodes a FabH homolog with significant homology to the *E. coli* enzyme, and because purified *P. aeruginosa* FabH was not required for in vitro FAS from acetyl-CoA and malonyl-CoA, the existence of and the necessity for β -ketoacyl-ACP synthase III in these organisms remains yet to be established.

Virtually nothing is known about the genetic regulation of the *fab* genes and many interesting questions can be asked. For example, why is the *P. aeruginosa fabI* gene the last gene in an oligopeptide permease-encoding operon? Is it subjected to the same regulatory mechanisms as the genes encoding the oligopeptide permease? One unique feature of *fab* gene organization in the three *Pseudomonas* genome sequences obtained thus far is that *fabA* and *fabB* are clustered, probably in the same transcriptional unit as has been established for *P. aeruginosa*. We have recently found that *fabB* expression in *P. aeruginosa* is highly upregulated in established, several days old biofilms (K. Sauer and H.P. Schweizer, unpublished observations), but the mechanism(s) underlying this *fabAB* operon upregulation and its biological significance remain to be established.

Lastly, the bacterial fatty acid biosynthetic pathway remains a viable and valuable target for antibacterial drug discovery since there are still many unexploited opportunities for the identification of compounds that specifically target bacterial FAS.

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REFERENCES

1. Aguilar, P.S., Cronan, J.E., and DeMendoza, D., 1998, A *Bacillus subtilis* gene induced by cold shock encodes a membrane phospholipid desaturase. *J. Bacteriol.*, 180:2194–2200.
2. Anderson, A.J., Haywood, G.W., and Dawes, E.A., 1990, Biosynthesis and composition of poly(hydroxyalkanoates). *Int. J. Biol. Macromol.*, 12:102–105.
3. Baldock, C., Rafferty, J.B., Sedelnikova, S.E., Baker, P.J., Stuitje, A.R., Slabas, A.R., Hawkes, T.R., and Rice, D.W., 1996, A mechanism of drug action revealed by structural studies of enoyl reductase. *Science*, 274:2107–2110.
4. Barry, C.E., Lee, R.E., Mdluli, K., Sampson, A.E., Schroeder, B.G., Slayden, R.A., and Yuan, Y., 1998, Mycolic acids: Structure, biosynthesis and physiological functions. *Prog. Lipid Res.*, 37:143–179.
5. Best, E. and Knauf, V.C., 1993, Organization and nucleotide sequence of the genes encoding the biotin carboxyl carrier protein and biotin carboxylase protein of *Pseudomonas aeruginosa* acetyl coenzyme A carboxylase. *J. Bacteriol.*, 175:6881–6889.
6. Bhargava, H.N. and Leonard, P.A., 1996, Triclosan: Applications and safety. *Am. J. Infect. Control*, 24:209–218.
7. Bloch, K., 1971, β -hydroxythioester dehydrase. In P.D. Boyer (ed.), *The Enzymes*. Academic Press, New York.
8. Campbell, J.W. and Cronan, J.E., 2001, Bacterial fatty acid biosynthesis: targets for antibacterial drug discovery. *Annu. Rev. Microbiol.*, 55:305–332.
9. Campos-Garcia, J., Caro, A.D., Najera, R., Miller-Maier, R.M., Al-Tahhan, R.A., and Soberon-Chavez, G., 1998, The *Pseudomonas aeruginosa* *rhlG* gene encodes an NADPH-dependent β -ketoacyl reductase which is specifically involved in rhamnolipid synthesis. *J. Bacteriol.*, 180:4442–4451.
10. Cha, C., Chen, Y.C., Shaw, P.D., and Farrand, S.K., 1998, Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. *Mol. Microbe Plant Interact.*, 11:1119–1129.
11. Chang, Y.-Y., Eichel, J., and Cronan, J.E., 2000, Metabolic instability of *Escherichia coli* cyclopropane fatty acid synthase is due to RpoH-dependent proteolysis. *J. Bacteriol.*, 182:4288–4294.
12. Chayabutra, C. and Ju, L.K., 2001, Polyhydroxyalkanoic acids and rhamnolipids are synthesized sequentially in hexadecane fermentation by *Pseudomonas aeruginosa*. *Biotechnol. Prog.*, 17:419–423.

13. Chin-A-Woeng, T.F., van den Broek, D., de Voer, G., van der Drift, K.M., Tuinman, S., Thomas-Oates, J.E., Lugtenberg, B.J., and Bloemberg, G.V., 2001, Phenazine-1-carboxamide production in the biocontrol strain *Pseudomonas chlororaphis* PCL1319 is regulated by multiple factors secreted into the growth medium. *Mol. Plant Microbe Interact.*, 14:969–979.
14. Chuanchuen, R., Karkhoff-Schweizer, R.R., and Schweizer, H.P., 2003, High-level triclosan resistance in *Pseudomonas aeruginosa* is solely due to efflux. *Am. J. Infect. Control*, 31:124–127.
15. Clark, D.P., DeMendoza, D., Polacco, M.L., and Cronan, J.E., 1983, β -hydroxydecanoyl thio ester dehydrase does not catalyze a rate-limiting step in *Escherichia coli* unsaturated fatty acid synthesis. *Biochemistry*, 22:5897–5902.
16. Cronan, J.E. and Rock, C.O., 1996, Biosynthesis of membrane lipids. In F.C. Neidhardt, R. Curtiss III, J.L. Ingraham, E.C.C. Lin, K. Brooks Low, B. Magasanik, W.S. Reznikoff, M. Riley, M. Schaechter, and H.E. Umbarger (eds), *Escherichia coli* and *Salmonella*, pp. 612–636. American Society for Microbiology Press, Washington, DC.
17. Crowfoot, P.D. and Hunt, A.L., 1970, Induced synthesis of cyclopropane fatty acid synthetase in *Pseudomonas fluorescens*. *Biochim. Biophys. Acta*, 218:555–557.
18. De Kievit, T.T. and Iglewski, B.H., 2000, Bacterial quorum sensing in pathogenic relationships. *Infect. Immun.*, 68:4839–4849.
19. Dees, S.B., Hollis, D.G., Weaver, R.E., and Moss, C.W., 1983, Cellular fatty acid composition of *Pseudomonas marginata* and closely associated bacteria. *J. Clin. Microbiol.*, 18:1073–1078.
20. Dotson, G.D., Kaltashov, I.A., Cotter, R.J., and Raetz, C.R.H., 1998, Expression cloning of a *Pseudomonas* gene encoding a hydroxydecanoyl-acyl carrier protein-dependent UDP-GlcNAc acyltransferase. *J. Bacteriol.*, 180:330–337.
21. Dubois-Brissonnet, F., Malgrange, C., Guerin-Mechin, L., Heyd, B., and Leveau, J.Y. 2001, Changes in fatty acid composition of *Pseudomonas aeruginosa* ATCC15442 induced by growth conditions: Consequences of resistance to quaternary ammonium compounds. *Microbios*, 106:97–110.
22. Ellis, R.J., Timms-Wilson, T.M., and Bailey, M.J., 2000, Identification of conserved traits in fluorescent pseudomonads with antifungal activity. *Environ. Microbiol.*, 2:274–284.
23. Epple, G., Van der Drift, K.M.G.M., Thomas-Oates, J.E., and Geiger, O., 1998, Characterization of a novel acyl carrier protein, RkpF, encoded by an operon involved in capsular polysaccharide biosynthesis in *Sinrhizobium meliloti*. *J. Bacteriol.*, 180:4950–4954.
24. Ernst, R.K., Yi, E.C., Guo, L., Lim, K.B., Burns, J.L., Hackett, M., and Miller, S.I., 1999, Specific lipopolysaccharide found in cystic fibrosis airway *Pseudomonas aeruginosa*. *Science*, 286:1561–1565.
25. Finking, R., Solsbacher, J., Konz, D., Schobert, M., Schaefer, A., Jahn, D., and Marahiel, M.A., 2002, Characterization of a new type of phosphopantetheinyl transferase for fatty acid and siderophore synthesis in *Pseudomonas aeruginosa*. *J. Biol. Chem.* 277:50293–50302.
26. Fischl, A.S. and Kennedy, E.P., 1990, Isolation and properties of acyl carrier protein phosphodiesterase of *Escherichia coli*. *J. Bacteriol.*, 172:5445–5449.
27. Geiger, O., Spaink, H.P., and Kennedy, E.P., 1991, Isolation of the *Rhizobium leguminosarum* NodF nodulation protein: NodF carries a 4'-phosphopantetheine prosthetic group. *J. Bacteriol.*, 173:2872–2878.
28. Grogan, D.W. and Cronan, J.E., 1997, Cyclopropane ring formation in membrane lipids. *Microbiol. Mol. Biol. Rev.*, 61:429–441.
29. Hata, T., Sano, Y., Matsumae, A., Kamio, Y., Nomura, S., and Sugawara, R. 1960, Study of new antifungal antibiotic. *J. Bacteriol.* (Japan), 15:1075–1077.
30. Hayashi, T., Yamamoto, O., Sasaki, H., Kawaguchi, A., and Okazaki, A. 1983, Mechanism of action of the antibiotic thiolactomycin inhibition of fatty acid synthesis of *Escherichia coli*. *Biochem. Biophys. Res. Comm.*, 115:1108–1113.

31. Hayashi, T., Yamamoto, O., Sasaki, H., and Okazaki, H., 1984, Inhibition of fatty acid synthesis by the antibiotic thiolactomycin. *J. Antibiot.*, 37:1456–1461.
32. Heath, R.J. and Rock, C.O., 1996, Roles of the FabA and FabZ β -hydroxyacyl-acyl carrier protein dehydratase in *Escherichia coli* fatty acid biosynthesis. *J. Biol. Chem.*, 271:27795–27801.
33. Heath, R.J. and Rock, C.O., 2000, A triclosan-resistant bacterial enzyme. *Nature*, 406:145–146.
34. Heath, R.J., Rubin, J.R., Holland, D.R., Zhang, E., Snow, M.E., and Rock, C.O., 1999, Mechanism of triclosan inhibition of bacterial fatty acid synthesis. *J. Biol. Chem.*, 274:11110–11114.
35. Heath, R.J., White, S.W., and Rock, C.O., 2001, Lipid biosynthesis as a target for antibacterial agents. *Prog. Lipid Res.*, 40:467–497.
36. Heath, R.J., Yu, Y.-T., Shapiro, M.A., Olson, E., and Rock, C.O., 1998, Broad spectrum antimicrobial biocides target the FabI component of fatty acid biosynthesis. *J. Biol. Chem.*, 273:30316–30320.
37. Heipieper, H.J., de Waard, P., van der Meer, P., Killian, J.A., Isken, S., de Bont, J.A., Eggingk, G., and de Wolf, F.A., 2001, Regiospecific effect of *cis-trans* isomerization of unsaturated fatty acids in the solvent-tolerant strain *Pseudomonas putida* S12. *Appl. Environ. Microbiol.*, 57:541–547.
38. Heipieper, H.J., Diefenbach, R., and Keweloh, H. 1992, Conversion of *cis* unsaturated fatty acids to *trans*, a possible mechanism for the protection of phenol-degrading *Pseudomonas putida* P8 from substrate toxicity. *Appl. Environ. Microbiol.*, 58:1847–1852.
39. Herman, D.C., Zhang, Y., and Miller, R.M., 1997, Rhamnolipid (biosurfactant) effects on cell aggregation and biodegradation of residual hexadecane under saturated flow conditions. *Appl. Environ. Microbiol.*, 63:3622–3627.
40. Hoang, T.T., Ma, Y., Stern, R.J., McNeil, M.R., and Schweizer, H.P., 1999, Construction and use of low-copy number T7 expression vectors for purification of problem proteins: Purification of *Mycobacterium tuberculosis* RmlD and *Pseudomonas aeruginosa* LasI and RhlI proteins, and functional analysis of RhlI. *Gene*, 237:361–371.
41. Hoang, T.T. and Schweizer, H.P., 1999, Characterization of the *Pseudomonas aeruginosa* enoyl-acyl carrier protein reductase: A target for triclosan and its role in acylated homoserine lactone synthesis. *J. Bacteriol.*, 181:5489–5497.
42. Hoang, T.T. and Schweizer, H.P., 1997, Fatty acid biosynthesis in *Pseudomonas aeruginosa*: Cloning and characterization of the *fabAB* operon encoding β -hydroxydecanoyl-acyl carrier protein dehydratase (FabA) and β -ketoacyl-acyl carrier protein synthase I (FabB). *J. Bacteriol.*, 179:5326–5332.
43. Hoang, T.T., Sullivan, S.A., Cusick, J.K., and Schweizer, H.P., 2002, β -ketoacyl acyl carrier protein reductase (FabG) activity of the fatty acid biosynthetic pathway is a determining factor of 3-oxo-homoserine lactone acyl chain lengths. *Microbiology*, 148:3849–3856.
44. Hoffmann, N., Steinbuechel, A., and Rehm, B.H., 2000, Polyhydroxyalkanoate synthesis in *Pseudomonas oleovorans* from simple carbon sources: Homologous functional expression of cryptic *phaG* establishes a transacylase-mediated pathway. *Appl. Microbiol. Biotechnol.*, 54:665–670.
45. Hoffmann, N., Steinbuechel, A., and Rehm, B.H. 2000, The *Pseudomonas aeruginosa phaG* gene product is involved in the synthesis of polyhydroxyalkanoic acid consisting of medium-chain length constituents from non-related carbon sources. *FEMS Microbiol. Lett.*, 184:253–259.
46. Holtwick, R., Meinhardt, F., and Keweloh, H., 1997, *Cis-trans* isomerization of unsaturated fatty acids: Cloning and sequencing of the *cti* gene from *Pseudomonas putida* P8. *Appl. Environ. Microbiol.*, 63:4292–4297.
47. Huijberts, G., de Rijk, T., de Waard, P., and Eggink, G., 1994, ^{13}C nuclear magnetic resonance studies of *Pseudomonas putida* fatty acid metabolic routes involved in poly(3-hydroxyalkanoate) synthesis. *J. Bacteriol.*, 176:1661–1666.

48. Issartel, J.P., Koronakis, V., and Hughes, C., 1991, Activation of *Escherichia coli* prohaemolysin to the mature toxin by acyl carrier protein-dependent fatty acylation. *Nature*, 351:759–761.
49. Jacques, N.A., 1981. Studies on cyclopropane fatty acid synthesis. Correlation between the state of reduction of respiratory components and the accumulation of methylene hexadecanoic acid by *Pseudomonas denitrificans*. *Biochim. Biophys. Acta*, 665:270–282.
50. Jacques, N.A. and Hunt, A., 1980, Studies on cyclopropane fatty acid synthesis. Effect of carbon source and oxygen tension on cyclopropane fatty acid synthetase activity in *Pseudomonas denitrificans*. *Biochim. Biophys. Acta*, 619:453–470.
51. Junker, F. and Ramos, J.L., 1999, Involvement of a *cis/trans* isomerase Cti in solvent resistance of *Pseudomonas putida* DOT-T1E. *J. Bacteriol.*, 181:5693–5700.
52. Kawai, Y., Yano, I., Kaneda, K., and Yabuuchi, E., 1988, Ornithine-containing lipids of some *Pseudomonas* species. *Eur. J. Biochem.*, 175:633–641.
53. Kearns, D.B., Robinson, J., and Shimkets, L.J., 2001, *Pseudomonas aeruginosa* exhibits directed twitching motility up phosphoethanolamine gradients. *J. Bacteriol.*, 183:763–767.
54. Keweloh, H. and Heipieper, H.J., 1996, *Trans* unsaturated fatty acids in bacteria. *Lipids*, 31:129–137.
55. Kremer, L., Douglas, J.D., Baulard, A.R., Morehouse, C., Guy, M.R. *et al.*, 2000, Thiolactomycin and related analogues as novel antimycobacterial agents targeting KasA and KasB condensing enzymes in *Mycobacterium tuberculosis*. *J. Biol. Chem.*, 275:16857–16864.
56. Kutchma, A.J., Hoang, T.T., and Schweizer, H.P., 1999, Characterization of a *Pseudomonas aeruginosa* fatty acid biosynthetic gene cluster: Purification of acyl carrier protein (ACP) and malonyl-coenzyme A:ACP transacylase (FabD). *J. Bacteriol.*, 181:5498–5504.
57. Laue, B.E., Jiang, Y., Chhabra, S.R., Jacob, S., Stewart, G.S., Hardman, A., Downie, J.A., O’Gara, F., and Williams, P., 2000, The biocontrol strain *Pseudomonas fluorescens* F113 produces the *Rhizobium* small bacteriocin, *N*-(3-hydroxy-7-*cis*-tetradecenoyl) homoserine lactone, via HdtS, a putative homoserine lactone synthase. *Microbiology*, 146:2469–2480.
58. Li, X.-Z., Zhang, L., and Poole, K., 1998, Role of the multidrug efflux systems of *Pseudomonas aeruginosa* in organic solvent tolerance. *J. Bacteriol.*, 180:2987–2991.
59. Loffeld, B., and Keweloh, H., 1996, *cis/trans* isomerization of unsaturated fatty acids as possible control mechanism of membrane fluidity in *Pseudomonas putida* P8. *Lipids*, 31:811–815.
60. Lopez-Lara, I.M. and Geiger, O., 2001, Novel pathway for phosphatidylcholine biosynthesis in bacteria associated with eukaryotes. *J. Biotechnol.*, 91:211–221.
61. Madison, L.L. and Huisman, G.W., 1999, Metabolic engineering of poly(3-hydroxy-alkanoates): From DNA to plastic. *Mol. Biol. Rev.*, 63:21–53.
62. McMurry, L.M., Oethinger, M., and Levy, S.B., 1998, Triclosan targets lipid synthesis. *Nature*, 394:531–532.
63. Miller, M.B. and Bassler, B.L., 2001, Quorum sensing in bacteria. *Annu. Rev. Microbiol.*, 55:165–199.
64. Miller, R.M., 1995. Biosurfactant-facilitated remediation of metal-contaminated soils. *Environ. Health Perspect.*, 103(Suppl.):59–62.
65. Miyakawa, S., Suzuki, K., Noto, T., Harada, Y., and Okazaki, H., 1982, Thiolactomycin, a new antibiotic. IV. Biological properties and chemotherapeutic activity in mice. *J. Antibiot.*, 35:411–419.
66. Mohan, S. and Raetz, C.R.H., 1994, Endotoxin biosynthesis in *Pseudomonas aeruginosa*: Enzymatic incorporation of laurate before 3-deoxy-D-manno-octulosonate. *J. Bacteriol.*, 176:6944–6951.
67. More, M.I., Finger, D., Stryker, J.L., Fuqua, C., Eberhard, A., and Winans, S.C., 1996, Enzymatic synthesis of a quorum-sensing autoinducer through use of defined substrates. *Science*, 272:1655–1658.
68. Moss, C.W. and Dees, S.B., 1976, Cellular fatty acids and metabolic products of *Pseudomonas* species obtained from clinical specimens. *J. Clin. Microbiol.*, 4:492–502.

69. Moss, C.W. and Dees, S.B. 1975, Identification of microorganisms by gas chromatographic-mass spectrometric analysis of cellular fatty acids. *J. Chromatogr.*, 112:594–604.
70. Nelson, K.E., Weinel, C., Paulsen, I.T., Dodson, R.J., Hilbert, H., Martins dos Santos, V.A.P., Fouts, D.E., Gill, S.R., Pop, M., Holmes, M., Brinkac, L., Beanan, M., DeBoy, R.T., Daugherty, S., Kolonay, J., Madupu, R., Nelson, W., White, D., Peterson, J., Khouri, H., Hance, I., Lee, P., Holtzapple, E., Scanlan, D., Tran, K., Moazzez, A., Utterback, T., Rizzo, M., Lee, L., Kosack, D., Moestl, D., Wedler, H., Lauber, J., Stjepandic, D., Hoheisel, J., Straetz, M., Heim, S., Kiewitz, C., Eisen, J., Timmis, K.N., Duesterhoeft, A., Tuemmler, B., and Fraser, C.M., 2002, Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ. Microbiol.*, 4:799–808.
71. Nishida, I., Kawaguchi, A., and Yamada, M., 1986, Effect of thiolactomycin on the individual enzymes of the fatty acid synthase system in *Escherichia coli*. *J. Biochem.*, 99:1447–1454.
72. Ochsner, U.A., Fiechter, A., and Reiser, J., 1994, Isolation, characterization, and expression in *Escherichia coli* of the *Pseudomonas aeruginosa* *rhlAB* genes encoding a rhamnosyltransferase involved in rhamnolipid biosurfactant synthesis. *J. Biol. Chem.*, 269:19787–19795.
73. Ochsner, U.A., Koch, A.K., Fiechter, A., and Reiser, J., 1994, Isolation and characterization of a regulatory gene affecting rhamnolipid synthesis in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 176:2044–2054.
74. Oishi, H., Noto, T., Sasaki, H., Suzuki, K., Hayashi, T., Okazaki, H., Ando, K., and Sawada, M., 1982, Thiolactomycin, a new antibiotic. I. Taxonomy of the producing organism, fermentation and biological properties. *J. Antibiot.*, 35:391–395.
75. Omura, S., 1976, The antibiotic cerulenin, a novel tool for biochemistry as an inhibitor of fatty acid synthesis. *Bacteriol. Rev.*, 40:681–697.
76. Parsek, M.R., Val, D.L., Hanzelka, B.L., Cronan, J.E., and Greenberg, E.P., 1999, Acyl homoserine-lactone quorum-sensing signal generation. *Proc. Natl. Acad. Sci. USA*, 98:4360–4365.
77. Passador, L., Cook, J.M., Gambello, M.J., Rust, L., and Iglewski, B.H., 1993, Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. *Science*, 260:1127–1130.
78. Payne, D.J., Miller, W.H., Berry, V., Brosky, J., Burgess, W.J., Chen, E., DeWolf, Jr., W.E., Fosberry, A.P., Greenwood, R., Head, M.S., Heerding, D.A., Janson, C.A., Jaworski, D.D., Keller, P.M., Manley, P.J., Moore, T.D., Newlander, K.A., Pearson, S., Polizzi, B.J., Qiu, X., Rittenhouse, S.F., Slater-Radosti, C., Salyers, K.L., Seefeld, M.A., Smyth, M.G., Takata, D.T., Uzinskas, I.N., Vaidya, K., Wallis, N.G., Winram, S.B., Yuan, C.C.K., and Huffman, W.F., 2002, Discovery of a novel and potent class of FabI-directed antibacterial agents. *Antimicrob. Agents Chemother.*, 46:3118–3124.
79. Pearson, J., Gray, K., Passador, L., Tucker, K., Eberhard, A., Iglewski, B., and Greenberg, E., 1994, Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proc. Natl. Acad. Sci. USA*, 91:197–201.
80. Pearson, J., Passador, L., Iglewski, B., and Greenberg, E. 1995, A second *N*-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA*, 92:1490–1494.
81. Pedrotta, V. and Witholt, B., 1999, Isolation and characterization of the *cis-trans* unsaturated fatty acid isomerase of *Pseudomonas oleovorans*. *J. Bacteriol.*, 181:3256–3261.
82. Pinkart, H.C. and White, D.C., 1997, Phospholipid biosynthesis and solvent tolerance in *Pseudomonas putida* strains. *J. Bacteriol.*, 179:4219–4226.
83. Qi, Q., Steinbuechel, A., and Rehm, B.H., 2000, In vitro synthesis of poly(3-hydroxydecanoate): Purification and enzymatic characterization of type II polyhydroxyalkanoate synthases PhaC1 and PhaC2 from *Pseudomonas aeruginosa*. *Appl. Microbiol. Biotechnol.*, 54:37–43.
84. Raetz, C.R.H. and Whitfield, C., 2002, Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.*, 71:635–700.

85. Rahim, R., Ochsner, U.A., Olvera, C., Graninger, M., Messner, P., Lam, J.S., and Soberon-Chavez, G., 2001, Cloning and functional characterization of the *Pseudomonas aeruginosa* *rhlC* gene that encodes rhamnosyltransferase 2, an enzyme responsible for di-rhamnolipid biosynthesis. *Mol. Microbiol.*, 40:708–718.
86. Ramos, J.L., Duque, E., Gallegos, M.T., Godoy, P., Ramos-Gonzalez, M.I., Rojas, A., Teran, W., and Segura, A., 2002, Mechanisms of solvent tolerance in Gram-negative bacteria. *Annu. Rev. Microbiol.*, 56:743–768.
87. Ramos, J.L., Duque, E., Rodriguez-Herva, J.-J., Godoy, P., Haidour, A., Reyes, F., and Fernandez-Barrero, A., 1997, Mechanisms for solvent tolerance in bacteria. *J. Biol. Chem.*, 272:3887–3890.
88. Rehm, B.H., Krueger, N., and Steinbüchel, A., 1998, A new metabolic link between fatty acid *de novo* synthesis and polyhydroxyalkanoic acid synthesis. *J. Biol. Chem.*, 273:24044–24051.
89. Rehm, B.H., Mitsky, T.A., and Steinbüchel, A., 2001, Role of fatty acid *de novo* biosynthesis in polyhydroxyalkanoic acid (PHA) and rhamnolipid synthesis by pseudomonads: Establishment of the transacylase (PhaG)-mediated pathway for PHA synthesis in *Escherichia coli*. *Appl. Environ. Microbiol.*, 67:3102–3109.
90. Rehm, B.H. and Steinbüchel, A., 1999, Biochemical and genetic analysis of PHA synthases and other proteins required for PHA synthesis. *Int. J. Biol. Macromol.*, 13:83–88.
91. Ren, Q., Sierro, N., Witholt, B., and Kessler, B., 2000, FabG, an NADPH-dependent 3-ketoacyl reductase of *Pseudomonas aeruginosa*, provides precursors for medium-chain-length poly-3-hydroxyalkanoate biosynthesis in *Escherichia coli*. *J. Bacteriol.*, 182:2978–2981.
92. Rocchetta, H.L., Burrows, L.L., and Lam, J.S., 1999, Genetics of O-antigen biosynthesis in *Pseudomonas aeruginosa*. *Microbiol. Mol. Biol. Rev.*, 63:523–553.
93. Schweizer, H.P., 1998, Intrinsic resistance to inhibitors of fatty acid biosynthesis in *Pseudomonas aeruginosa* is due to efflux: Application of a novel technique for generation of unmarked chromosomal mutations for the study of efflux systems. *Antimicrob. Agents Chemother.*, 42:394–398.
94. Slayden, R.A., Lee, R.E., and Barry, C.E., 2000, Isoniazid affects multiple components of the type II fatty acid synthase system of *Mycobacterium tuberculosis*. *Mol. Microbiol.*, 38:514–525.
95. Smith, R.S. and Iglewski, B.H., 2003, *P. aeruginosa* quorum-sensing and virulence. *Curr. Opin. Microbiol.*, 6:56–60.
96. Stanghellini, M.E. and Miller, R.M., 1997, Biosurfactants: Their identity and potential efficiency in the biological control of zoospore plant pathogens. *Plant Dis.*, 81:4–12.
97. Steidle, A., Allesen-Holm, M., Riedel, K. Berg, G., Gibskov, M., Molin, S., and Eberl, L., 2002, Identification and characterization of an *N*-acylhomoserine lactone-dependent quorum-sensing system in *Pseudomonas putida* strain IsoF. *Appl. Environ. Microbiol.*, 68:6371–6382.
98. Stover, C.K., Pham, X.-Q., Erwin, A.L., Mizoguchi, S.D., Warrenner, P., Hickey, M.J., Brinkman, F.S., L., Hufnagle, W.O., Kowalik, D.J., Lagrou, M., Garber, R.L., Goltry, L., Tolentino, E., Westbrook-Wadman, S., Yuan, Y., Brody, L.L., Coulter, S.N., Folger, K.R., Kas, A., Larbig, K., Lim, R., Spencer, D. Wong, G.K.-S., Wu, Z., Paulsen, I.T., Reizer, J., Saier, M.H., Hancock, R.E.W., Lory, S., and Olson, M.V. 2000, Complete genome sequence of *Pseudomonas aeruginosa*, an opportunistic pathogen. *Nature*, 406:959–964.
99. Timm, A. and Steinbüchel, A., 1992, Cloning and molecular analysis of the poly(3-hydroxyalkanoates acid) gene locus of *Pseudomonas aeruginosa* PAO1. *Eur. J. Biochem.* 209:15–30.
100. Val, D.L. and Cronan, J.E. 1998, In vivo evidence that *S*-adenosylmethionine and fatty acid intermediates are the substrates for the LuxI family of autoinducer synthases. *J. Bacteriol.*, 180:2644–2651.

101. Wang, A.Y. and Cronan, J.E., 1994, The growth phase-dependent synthesis of cyclopropane fatty acids in *Escherichia coli* is the result of an RpoS(KatF)-dependent promoter plus enzyme instability. *Mol. Microbiol.*, 11:1009–1017.
102. Wang, A.Y., Grogan, D.W., and Cronan, J.E. 1992, Cyclopropane fatty acid synthase of *Escherichia coli*: Deduced amino acid sequence. purification, and studies of the enzyme active site. *Biochemistry*, 31:11020–11028.
103. Watson, W.T., Minogue, T.D., Val, D.L., Beck Von Bodman, S., and Churchill, M.E.A., 2002, Structural basis and specificity of acyl-homoserine lactone signal production in bacterial quorum sensing. *Mol. Cell*, 9:685–694.
104. Wilderman, P.J., Vasil, A.I., Martin, W.E., Murphy, R.C., and Vasil, M.L., 2002, *Pseudomonas aeruginosa* synthesizes phosphatidylcholine by use of the phosphatidylcholine synthase pathway. *J. Bacteriol.*, 184:4792–4799.
105. Winder, F., 1982, Mode of action of antimycobacterial agents and associated aspects of the molecular biology of the mycobacteria. In C. Ratledge and J.L., Stanford (eds), *The Biology of Mycobacteria*. Vol. 1, pp. 354–441, Academic Press, San Diego.
106. Wood, D.W., Gong, F., Daykin, M.M., Williams, P., and Pierson, L.S. 1997, N-acyl-homoserine lactone-mediated regulation of phenazine gene expression in *Pseudomonas aureofaciens* 30-84 in the wheat rhizosphere. *J. Bacteriol.*, 179:7663–7670.
107. Wyckoff, T.J.O., Lin, S., Cotter, R.J., Dotson, G.D., and Raetz, C.R.H. 1998, Hydrocarbon rulers in UDP-*N*-acetylglucosamine acyltransferases. *J. Biol. Chem.*, 273:32369–32372.

THE BIOSYNTHESIS OF HEMES, SIROHEME, VITAMIN B₁₂ AND LINEAR TETRAPYRROLES IN PSEUDOMONADS

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1. INTRODUCTION

Structure and function of tetrapyrroles. Tetrapyrroles are characterized by their four five-membered pyrrole rings usually linked together via single atom bridges (Figure 1). The four rings of the macrocycle are labeled clockwise A–D starting with the first of the three symmetric rings with regard to the ring substituents. Two principal classes of cyclic tetrapyrroles are found in pseudomonads. The porphyrins, including various hemes, are characterized by their completely saturated ring system. The porphinooids are more reduced cyclic tetrapyrroles and include vitamin B₁₂ (corrinoids), siroheme and heme *d*₁. In cyclic tetrapyrroles, the nitrogen atoms of the four pyrrole rings are used to chelate a variety of divalent cations. Tetrapyrroles are very distinct in color. The pink cobalt-containing vitamin B₁₂ derivatives are the most complex known tetrapyrroles¹. They are involved in complex enzymatic reactions like radical-dependent nucleotide reduction, rearrangements and methyl transfer.

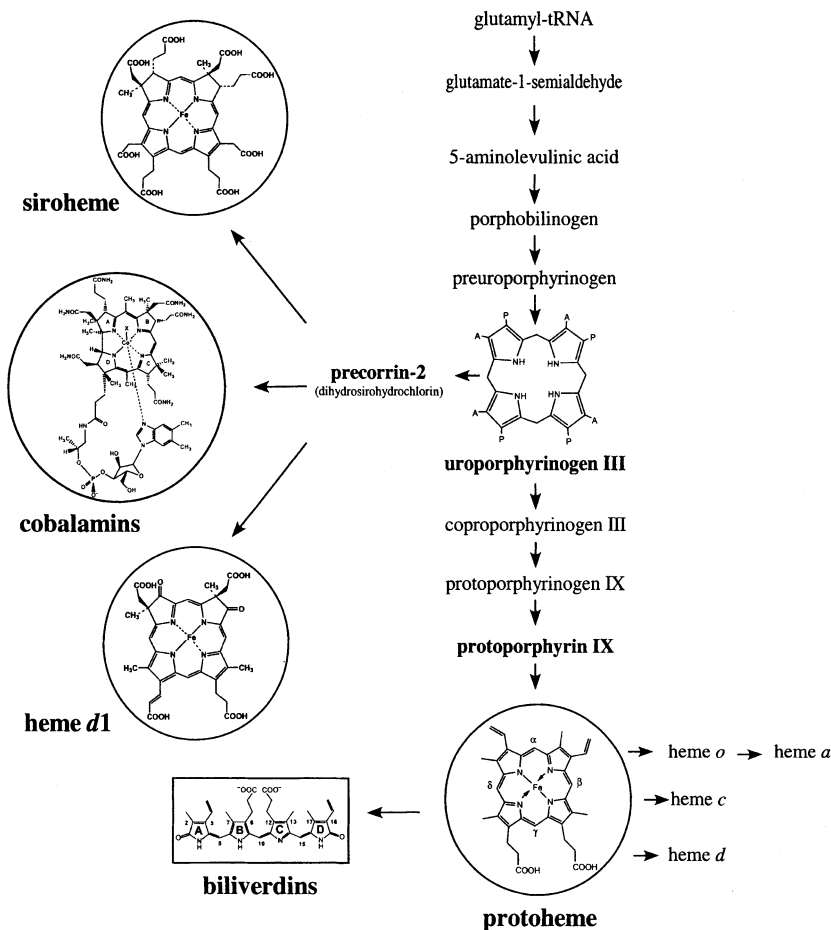


Figure 1. Overview of the biosynthesis of tetrapyrroles in microorganisms. In pseudomonads, all tetrapyrroles are synthesized via the C5-pathway starting from glutamyl-tRNA. The six biologically important porphyrin- or porphinoind-based tetrapyrroles are shown in circles. Biliverdin IX α is representatively shown for the group of linear tetrapyrroles (box).

The iron-chelated siroheme is required for the six electron reduction reactions during assimilatory nitrite or sulphite reduction². The green pigment heme d_1 , is part of the dissimilatory nitrite reductase in pseudomonads that differs significantly in structure and color from the otherwise porphyrin-based hemes³.

Heme, an iron-containing porphyrin, serves multiple cellular functions. As a prosthetic group of proteins, it is involved via its electron transfer capacity, in the metabolism of molecular oxygen, other diatomic gases and in multiple redox reactions⁴. Exogenous heme serves as an iron supply for many

pathogenic bacteria that colonize eukaryotic cells^{5, 6}. Heme also acts as a regulatory molecule, modulating gene expression at the transcriptional, translational and protein stability level⁷⁻⁹. In prokaryotes, cytochromes are the most abundant heme containing proteins. Cytochromes are electron transfer proteins and are involved in the final reduction of oxygen in aerobic respiration. Interestingly, the requirement for heme is not restricted to aerobic metabolism. Anaerobic respiring systems which employ alternative electron acceptors like nitrate also utilize electron transferring hemes¹⁰. Indeed, *Pseudomonas aeruginosa* synthesizes most of its heme under anaerobic denitrifying conditions¹¹. Various different types of hemes including heme *a*, heme *b*, heme *c* and heme *o* have been described for pseudomonads¹².

The other tetrapyrrolic structure found in pseudomonads are the open-chain molecules which are all derived from heme by oxidative cleavage^{13, 14}. However, their nomenclature is not consistent with that of the porphyrin system. The various rings of linear tetrapyrroles are likewise labeled A–D but starting from cleavage position of the heme macrocycle (Figure 1).

2. BIOSYNTHESIS OF HEME

2.1. The tRNA-Dependent Biosynthesis of 5-Aminolevulinic Acid

The common precursor of all tetrapyrroles, 5-aminolevulinic acid (ALA) is synthesized in nature by two alternative routes called the 'Shemin pathway'¹⁵ and the C₅-pathway^{16, 17}. In humans, animals, fungi and the α -group of proteobacteria, ALA synthase catalyzes the condensation of succinyl coenzyme A and glycine to ALA with the release of carbon dioxide and coenzyme A^{18, 19}. However, in *P. aeruginosa*, *Pseudomonas stutzeri* and *Pseudomonas putida*, ALA formation via the C₅-pathway has been demonstrated²⁰ (Figure 2). This route, which is found in most bacteria, all archaea and plants, involves the incorporation of the C₅-skeleton of glutamate into ALA. This transformation is mediated via glutamyl-tRNA, which is more commonly associated with translation and protein synthesis. Initially, the glutamyl-tRNA is reduced to glutamate-1-semialdehyde (GSA) by an NADPH-dependent glutamyl-tRNA reductase (GluTR)¹⁷. In the following reaction, glutamate-1-semialdehyde-2,1-aminomutase (GSAM) transaminates GSA in a pyridoxamine 5'-phosphate (PMP)-dependent reaction to form ALA^{21, 22}. The only known example of an organism in which both pathways exist in parallel is the phytoflagellate *Euglena gracilis*²³.

Glutamyl-tRNA reductase is a unique enzyme as its substrate, glutamyl-tRNA simultaneously participates in both protein and tetrapyrrole biosynthesis^{17, 24}. Biochemical and crystallographic data for the recombinant GluTR

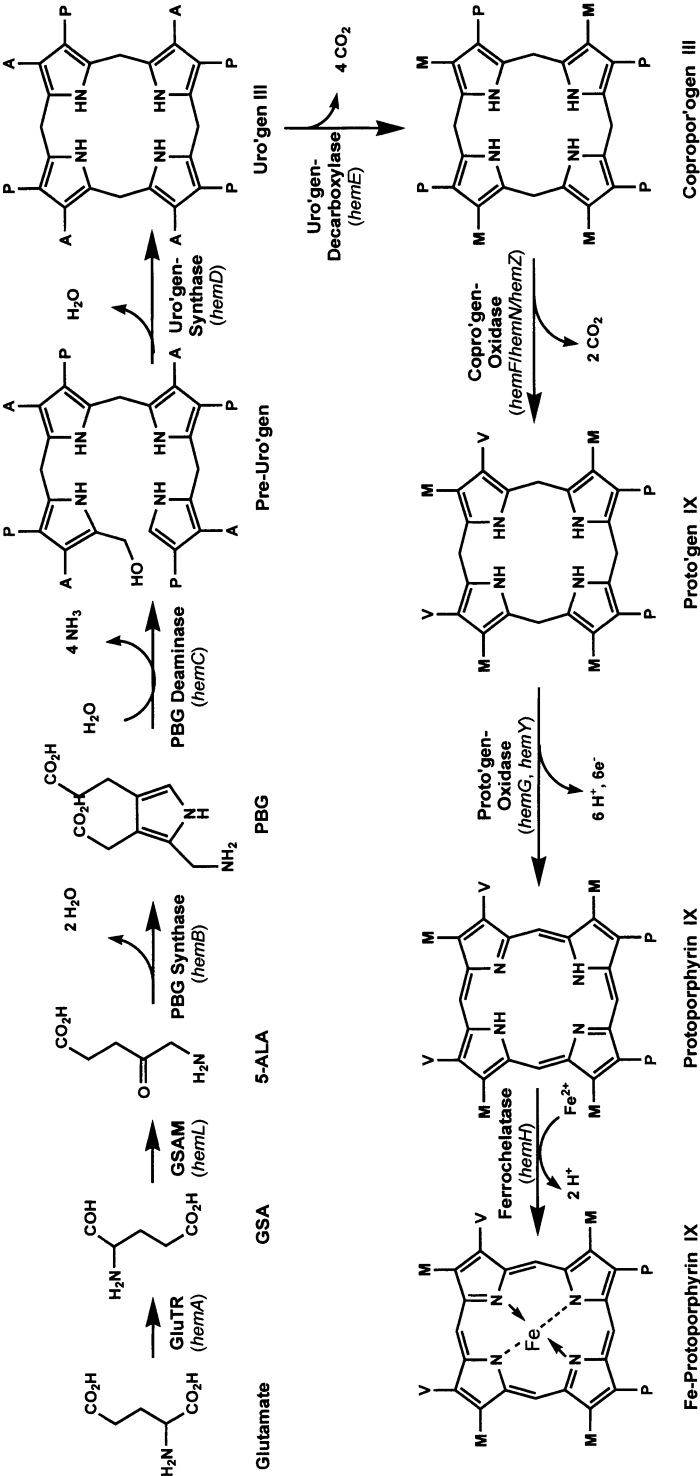


Figure 2. Biosynthesis of heme. The biosynthesis of heme from glutamate to Fe-protoporphyrin is shown with all proteins involved and the encoding genes.

from the extreme thermophilic archaeon *Methanopyrus kandleri*, led to the elucidation of the catalytic mechanism and its structural basis^{25–27}. The thiolate of an active site cysteine residue acts as a nucleophile and attacks the α -carbonyl group of tRNA-bound glutamate forming an enzyme-bound thioester intermediate with the concomitant release of tRNA^{Glu} (Figure 3A). This reaction intermediate was isolated and visualized for *Escherichia coli* GluTR²⁸. Hydride transfer from NADPH to the thioester-bound glutamate produces glutamate-1-semialdehyde. The crystal structure of *M. kandleri* GluTR has been solved and revealed an unusual extended V-shaped dimer²⁶ (Figure 3B). Each monomer consists of three distinct domains, an N-terminal catalytical domain, an NADPH-binding domain and a C-terminal dimerization domain, arranged along an extended curved ‘spinal’ α -helix (Figure 3B). The crystal structure of GluTR was solved in complex with the competitive inhibitor glutamycin²⁵, representing the 3'-terminal adenosine residue of the tRNA where the bridging oxygen of the aminoacyl-group was replaced by nitrogen to form a stable amide bond. The structure of the enzyme–inhibitor complex supports the proposed catalytic mechanism^{25, 26, 28}.

The second step of ALA formation requires the transfer of the C2 amino group of GSA to the C1 position. This reaction is catalyzed by PMP-dependent GSAM (E.C. 5.4.3.8) which is encoded by the *hemL* gene in *P. aeruginosa*²². Although the reaction differs from a classical aminotransferase reaction by its intramolecular nature, GSAMs represent typical aminotransferases in catalysis and structure. Due to the nature of the pyridoxal 5'-phosphate (PLP)/PMP-cofactor of the enzyme, two catalytic paths are conceivable. Starting with the PLP form of GSAM, reaction with GSA results in the formation of dioxo-valerate, while the PMP form leads to a 4,5-diaminovalerate (DAVA) intermediate. Recombinant GSAMs from *E. coli*, *Synechococcus* and other sources were found to catalyze both reactions^{21, 29}. Intensive kinetic investigations of *Synechococcus* and pea GSAM eventually identified DAVA as the true intermediate and the PMP-dependent reaction as the relevant path^{30, 31}. For various GSAMs, an active site lysine residue responsible for Schiff-base formation with PMP was experimentally identified^{21, 32}. The three-dimensional structure of GSAM reveals that the protein belongs to the extensively studied family of structurally related PLP-dependent proteins³³, which includes aminotransferases, mutases, synthetases, decarboxylases and racemases. The dimeric protein has an overall ellipsoidal shape when viewed along the dimer axis. Morphologically no distinct domains are apparent. The monomers interact through a large, convoluted interface. The PMP-cofactor and the substrate GSA are bound at the monomer–monomer interface. The crystal structure revealed the imperfect symmetry of the GSAM dimer. A loop essential for proper enzyme function laterally covers the substrate pocket and is partly disordered in one monomer, while it is well structured in the second³⁴.

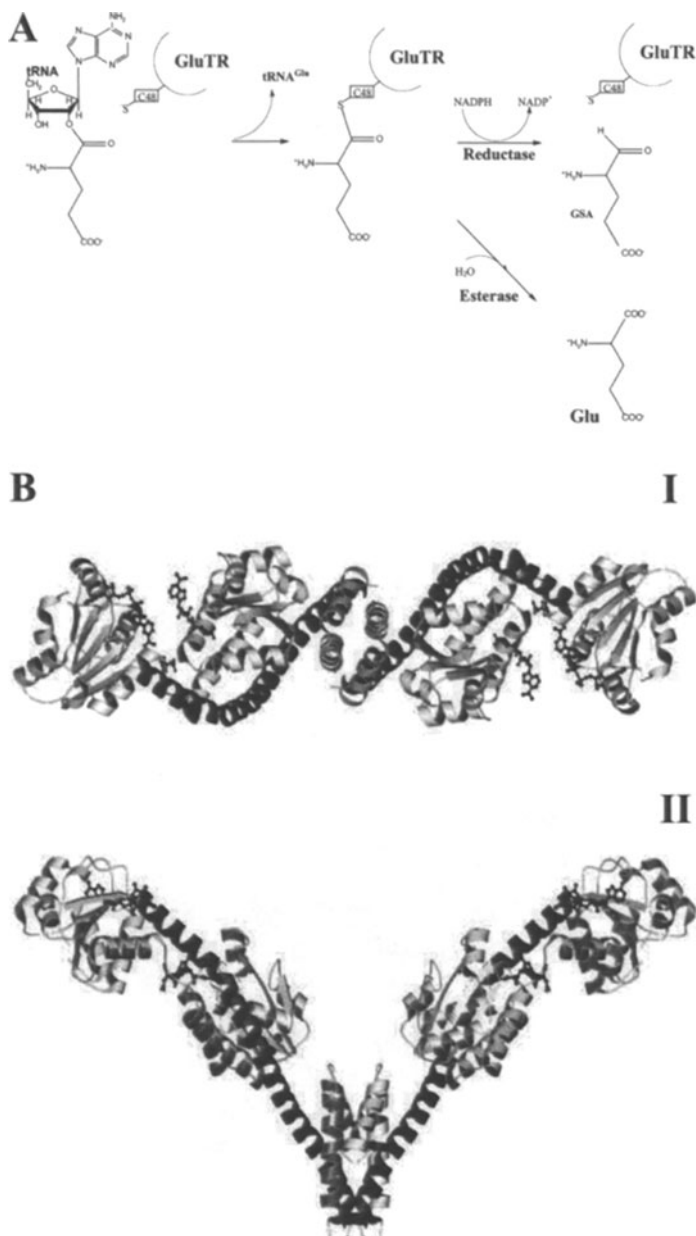


Figure 3. The first step of tetrapyrrole biosynthesis. A. Thioester-dependent mechanism of GluTR. The reactive cysteine residue nucleophilically attacks the aminoacyl bond of glutamyl-tRNA. An enzyme-localized thioester intermediate is formed with the release of free tRNA^{Glu}. The thioester is reduced by hydride transfer from NADPH leading to GSA. In the absence of NADPH, the reactive thioester bond is hydrolyzed and glutamate is liberated. B. A schematic diagram of the *M. kandleri* GluTR dimer viewed along the 2-fold-axis (I) and viewed perpendicular to the two-fold-axis (II).

Presumably, GSAM oscillates between two conformational states in which one monomer is in the closed, active state (with ordered active-site lid), while the second is in a relaxed state, allowing product and substrate to diffuse out of and into the active site, respectively.

2.2. Porphobilinogen Synthase

The first step common to the biosynthesis of all modified tetrapyrroles is the formation of the monopyrrole-derivative, porphobilinogen (PBG), which is catalyzed by porphobilinogen synthase (PBGS; aminolevulinic acid dehydratase; E.C. 4.2.1.24)³⁵ (Figure 2). The reaction involves the asymmetric condensation of two molecules of ALA. The two ALA substrate molecules are referred to as A- and P-side ALA due to the acetic- and propionic-acid side chains they contribute to the product. The corresponding ALA binding sites of PBGS are termed A-site and P-site, respectively. In *P. aeruginosa*, the *hemB* gene encodes PBGS which is an octameric enzyme of identical subunits^{36, 37}. Each of the eight PBGS subunits consists of an ($\alpha\beta$)₈-barrel (Figure 4A). The active site located in each subunit is close to the C-terminal end of the β -barrel. A flexible loop covers the active site and separates it from the surrounding medium. Common to all PBGSs is an N-terminal extension that is involved in subunit-subunit interaction^{38–40}. The formation of PBG involves the sequential formation of a C–C- and C–N-bond, respectively. The C–C-bond is formed by an aldol condensation whereas the C–N-bond is build via a Schiff-base. The crystal structures of several *P. aeruginosa* PBGS variants in combination with inhibitors strongly suggests that the synthesis of PBG involves a double Schiff-base mechanism^{40, 41}. The reaction starts with Schiff-base formation of the P-side ALA with a lysine residue in the active site, followed by A-side ALA binding to a second active site lysine. The removal of a hydrogen from C3 of the A-side ALA leads to the A-side enamine form. In the following step, the C–C-bond between the two ALA molecules is formed in an aldol-like addition. Through a Schiff-base exchange reaction, the C–N-bond between A- and P-side ALA is formed. After proton transfer and abstraction, the product is aromatized and released⁴¹ (Figure 4B). All PBGS contain a metal-binding site at the active site that consists of either a cysteine-rich sequence (DXCXCX(Y/F)X₃G(H/Q)CG) or an aspartate-rich region (DXALDX(Y/F)X₃G(H/Q)DG). The cysteine rich region is involved in zinc coordination. Enzymes that contain the aspartate-rich region do not use zinc but utilize magnesium and/or potassium instead. Besides the metal-binding region at the active site, many PBGS also contain an allosteric metal binding region (RX_{~164}DX_{~65}EXXXD)^{42, 43}. If present, the allosteric magnesium ion is involved in opening and closing a lid that covers the active site of each monomer⁴⁰. Since this complex metal-dependence led to some confusion in the literature in the past, a novel classification for PBGS enzymes has been

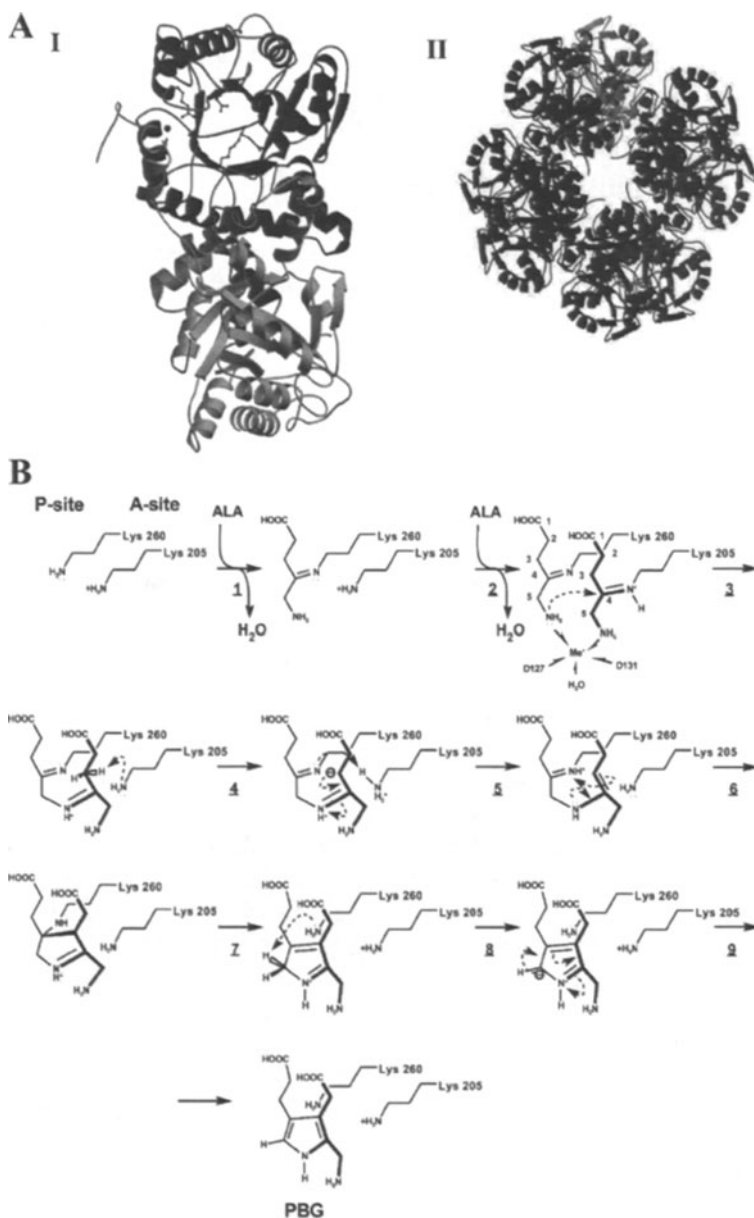


Figure 4. Structure and catalytic mechanism of *P. aeruginosa* PBGS. A. Ribbon diagram showing the fold of *P. aeruginosa* PBGS dimer viewing toward the active site pocket of monomer A (I); Structure of the PBGS octamer composed of four dimers projecting down the crystallographic 4-fold axis (II). B. Postulated catalytic mechanism of *P. aeruginosa* PBGS. The steps in the condensation of A- and P-side ALA to form PBG are indicated by numbers: **1** Binding of P-side ALA; **2** Binding of A-side ALA; **3** Abstraction of H⁺ from C3 of A-side ALA, yielding the A-site enamine; **4** Aldole addition forming the C-C-bond between A- and

proposed by Jaffe⁴³. This new designation divides PBGS enzymes into four groups on the basis of an active site zinc and an allosteric magnesium.

2.3. Porphobilinogen Deaminase

Porphobilinogen deaminase (PBGD; hydroxymethylbilane synthase) (EC 4.3.1.8) catalyzes the stepwise polymerization of four PBG molecules to the unstable linear tetrapyrrole preuroporphyrinogen (1-hydroxymethylbilane). During catalysis, the growing tetrapyrrole chain is covalently bound to the enzyme via a unique cofactor consisting of two molecules PBG⁴⁴ (Figure 5A). This dipyrromethane cofactor, itself covalently tethered to the enzyme by a thioether linkage, acts as a primer in the tetramerization of PBG but is not enzymatically integrated into the final product. During catalysis, one molecule of PBG is added per reaction cycle with the loss of one molecule ammonia until an enzyme-bound hexapyrrole is formed. Each coupling step involves two sequential chemical reactions. First, the substrate PBG is deaminated followed by a nucleophilic attack by the α -carbon of the terminal ring of the enzyme-bound cofactor or the substrate cofactor-complex. Next, a hydrogen is removed from the α -position. The overall reaction is repeated four times. During the final step, a water molecule attacks the tetrapyrrolic azafulvene to yield pre-uroporphyrinogen⁴⁵.

Several crystallographic structures of PBGD have previously been determined^{46–48}. The protein consists of three equal sized domains, the N-terminal, central- and C-terminal domains. The active site cavity is designed that the available space accommodates approximately $3\frac{1}{2}$ pyrrole rings. This limitation explains why the elongation does not proceed beyond the tetrapyrrolic product. PBGD is a monomeric enzyme and is encoded by the *P. aeruginosa hemC* gene. The *hemC* gene forms an operon with the *hemD* gene encoding the enzyme that catalyzes the subsequent step⁴⁹. This genomic arrangement might reflect that the product of the PBDG reaction, pre-uroporphyrinogen, is very unstable and will spontaneously cyclize to the toxic type I isomer. Therefore, the presence of the next enzyme in the pathway, uroporphyrinogen III synthase, prevents the accumulation of preuroporphyrinogen.

2.4. Uroporphyrinogen III Synthase

Uroporphyrinogen III synthase (Cosynthase; E.C. 4.2.1.75) is a comparatively small enzyme with a molecular mass of ~26 kDa that catalyzes the cyclization of pre-uroporphyrinogen with the inversion of ring D to produce the first asymmetric molecule in the pathway, uroporphyrinogen III.

Figure 4. Continued.

P-side-ALA; 5–7 Schiff-base exchange producing a C–N bond between A- and P-side ALA; 8 Transfer of H⁺ to Lys260; 9 *Trans*-elimination of P-site lysine; abstraction of the *pro*-RH⁺ from C5 of P-side ALA, aromatization and release of PBG.

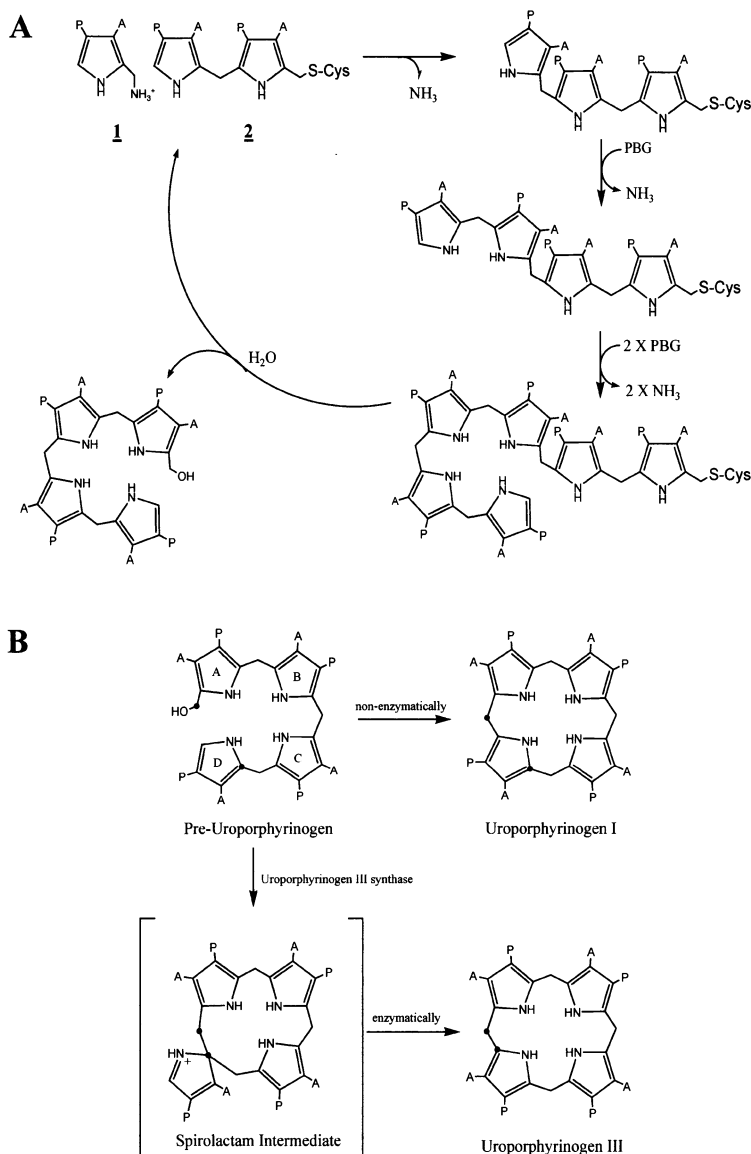


Figure 5. Unique catalytic mechanisms of two tetrapyrrole biosynthetic enzymes. A. Tetrapolymerization reaction catalyzed by PBGD. **1**, substrate PBG; **2**, dipyrromethane cofactor. The dipyrromethane cofactor is covalently attached to the protein via a cysteine residue. During each step one molecule of PBG is attached with the release of ammonia. The final product preuoporphyrinogen is hydrolytically released from the protein (see text for details). B. Spiro-lactam intermediate in the biosynthesis of uroporphyrinogen III. Non-enzymatically pre-uroporphyrinogen is cyclized to the toxic uroporphyrinogen I. Uroporphyrinogen III synthase converts pre-uroporphyrinogen via an spirocyclic pyrrolene intermediate with inversion of the D ring to yield the asymmetric uroporphyrinogen III.

Mechanistic studies have shown that the formation of uroporphyrinogen III involves an electrophilic addition of the substrate's hydroxymethyl group to C-16. This results in the cleavage of the C-15 to C-16 bond (Figure 5B). This mechanism is referred to as the spiro-mechanism since the key intermediate is a spiro-pyrrolenine produced by the initial cyclization (Figure 5B). The amino acid sequences for uroporphyrinogen III synthases from different organisms show little if any homology. Most conserved amino acid residues were localized via X-ray crystallography of the human enzyme in a large open cleft located between two central domains. This region of uroporphyrinogen III synthase seems to accommodate the active site⁵⁰. The observed structure provides clear evidence for interdomain flexibility. This suggests that a considerable conformational change may be required during catalysis^{50, 51}.

2.5. Uroporphyrinogen Decarboxylase

Uroporphyrinogen decarboxylase (URO-D; E.C.4.1.1.37) catalyzes the sequential decarboxylation of the four acetate side chains of uroporphyrinogen III localized at C-2, C-7, C-12 and C-18 to yield methyl groups with the formation of coproporphyrinogen III⁵². URO-D is a homodimeric protein consisting of two subunits that fold into a distorted ($\alpha\beta$)₈-barrel^{53, 54}. URO-D appears to be the first known decarboxylating enzyme with no cofactor or prosthetic group requirement⁵⁵. At physiological substrate concentrations, the decarboxylation occurs in an ordered manner, starting with the acetate side chain of the asymmetric ring (ring D). Although crystal structures of the enzyme are available^{53, 54}, there is still a great lack of understanding of the exact catalytic mechanisms. This is especially intriguing as a 180° flip of the first reaction intermediate is required to bring the side chains of ring A into the active site that was previously occupied with the side chains of ring D. A dimer-dependent mechanism has been proposed in which the rate limiting decarboxylation of ring D⁵⁶ occurs at the active site of one monomer while the other three decarboxylations may take place at the catalytic cleft of the other monomer⁵³. The enzyme of *P. aeruginosa* is encoded by the *hemE* gene⁴⁹.

2.6. Coproporphyrinogen III Oxidases

The oxidative decarboxylation of coproporphyrinogen III (coprogen) to protoporphyrinogen IX (protopogen) is catalyzed by different types of coproporphyrinogen III oxidases (CPO, EC 1.3.3.3). CPO consecutively converts the propionate side chains of rings A and B to their corresponding vinyl groups⁵². Molecular oxygen serves as the electron acceptor for the oxidative decarboxylation reaction under aerobic conditions. In the absence of oxygen, alternative electron acceptors are utilized for catalysis. Therefore, at least two different

types of enzymes for coprogen oxidation are found in nature, one for the oxygen-dependent (HemF) and another for the oxygen-independent reaction (HemN, HemZ).

Most available data on the oxygen-dependent CPO (E.C. 1.3.3.3) are for the enzymes from eukaryotic sources⁵⁷. Human oxygen-dependent CPO is a dimer of identical subunits with no detectable metal ions or cofactors. Nevertheless, molecular oxygen is required for catalysis. Recently, a structural model for CPO based on the structure of urate oxidase has been proposed⁵⁸. Both enzymes are similar with regard to the catalytically required molecular oxygen and the observed lack of detectable cofactors or metal ions^{58, 59}.

Until recently, much less was known about the oxygen-independent CPO that is encoded by the *hemN* gene. Some bacteria carry a second *hemN*-like gene termed *hemZ*. The gene product shows significant amino acid sequence homology to HemN and was shown to be involved in oxygen-independent coproporphyrinogen III oxidation⁶⁰. There are no obvious similarities at the amino acid sequence level between oxygen-dependent HemF and the oxygen-independent CPOs HemN and HemZ. Purified HemN requires S-adenosylmethionine (SAM) and both an electron-donor and -acceptor for catalysis.

An oxygen-sensitive [4Fe-4S]-cluster coordinated by the Cysteine residues C62, C66 and C69 was identified by absorption spectroscopy and iron analysis⁶¹. Observed functional properties in combination with a recently published computer-based enzyme classification identified HemN as a 'Radical SAM enzyme'^{61, 62}. A radical mechanism for the oxidative decarboxylation of coproporphyrinogen III by HemN was recently postulated⁶¹ (Figure 6). This mechanism involves the reduction of the [4Fe-4S]-cluster from the +2 to the +1 state. This is followed by a homolytic cleavage of SAM to methionine and the formation of a 5'-deoxyadenosyl radical. This radical abstracts a hydrogen atom from the β -C atom of the propionate side chain of the substrate and generates the corresponding substrate radical. During the final reaction step, the vinyl group is formed and CO₂ is released. This last step requires an electron acceptor whose nature remains to be identified. The oxygen-dependent regulation of the *P. aeruginosa* *hemF* and *hemN* genes is described below⁶³.

2.7. Protoporphyrinogen Oxidase

Protoporphyrinogen oxidase (PPO; E.C. 1.3.3.4) catalyzes the six electron oxidation of protoporphyrinogen IX to form protoporphyrin IX. The result of this oxidation reaction is a conjugated double system of the tetrapyrrole macrocycle. Best characterized are the flavin-containing PPOs from eukaryotes⁶⁴. Molecular oxygen serves as terminal electron acceptor. Protoporphyrinogen oxidation in prokaryotes is far from being understood. Three different genes encoding putative bacterial PPOs have been described during

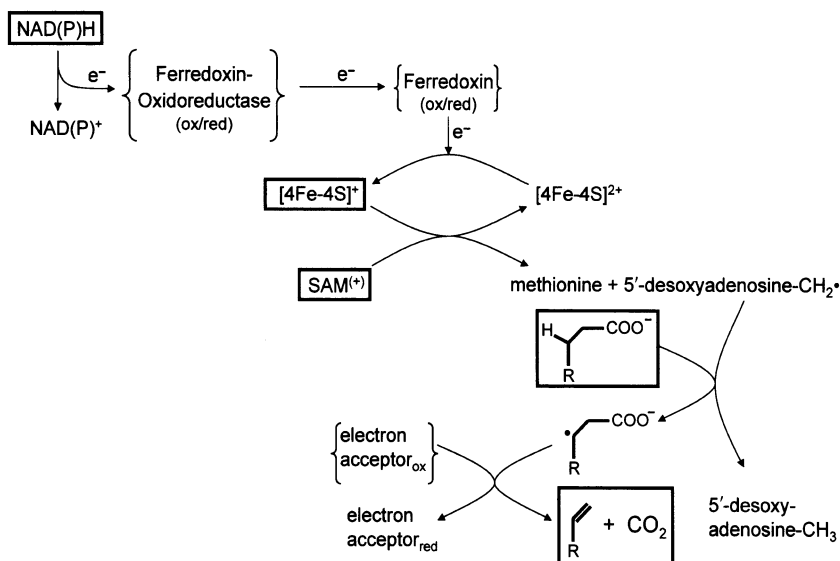


Figure 6. Postulated radical mechanism of oxygen independent coproporphyrinogen III oxidase. The homolytic cleavage of SAM generates a 5'-deoxyadenosyl radical which subsequently abstracts a hydrogen atom from the propionate side chain of the substrate to yield 5'-deoxyadenosine and a substrate radical. Electron donor for this step is the enzymes [4Fe-4S]⁺ cluster. In the next step, the product's vinyl group is formed through the elimination of CO₂. This step requires an electron acceptor to collect the remaining electron of the substrate radical⁶¹. Boxed compounds represent identified components, whereas compounds in parentheses represent activities still unknown.

the last decades: *hemG*, *hemK* and *hemY*^{65–67}. *E. coli hemK* was originally identified in a genetic screen for heme biosynthetic mutants⁶⁶. However, in 1999, HemK was biochemically identified as a methyltransferase^{68, 69}.

The *E. coli hemG* gene was first identified by the accumulation of protoporphyrinogen by an appropriate *hemG* mutant. The heme auxotrophic phenotype was rescued by the expression of the appropriate *hemG* or an eukaryotic PPO gene *in trans*^{65, 70}. So far, *hemG* genes are limited to six genera within the γ -group of proteobacteria and to *Mesorhizobium loti* an α -group proteobacterium. Currently, the known genomes of pseudomonads do not contain obvious *hemG* analogs. Therefore, the *hemG* gene might be unique to these few bacteria, contributing to a larger multienzyme complex in a species-specific manner⁴.

In the gram-positive soil bacterium *Bacillus subtilis*, the *hemY* gene encodes a PPO that is similar to the eukaryotic enzymes. HemY contains a flavin cofactor and utilizes molecular oxygen as a terminal electron acceptor^{71, 72}. Again, no obvious homologue of *hemY* was detected in pseudomonad

genomes. In agreement with these observations, *hemG* or *hemY* genes are not usually found in heme synthesizing prokaryotes. Among 28 heme synthesizing genera, 14 do not contain a recognizable PPO gene (*hemG* or *hemY*)⁷³. These observations imply that a so far unknown locus encodes a novel type of PPO. Alternatively, an already known protein might carry an additional enzymatic activity.

2.8. Ferrochelatase

The last enzymatic step of heme biosynthesis is the insertion of iron into the tetrapyrrole macrocycle of protoporphyrin IX. This reaction is catalyzed by ferrochelatase (E.C. 4.99.1.1; protoheme ferrolyase). The enzyme in pseudomonads is encoded by the *hemH* gene. The three-dimensional structure of the monomeric *B. subtilis* enzyme has been determined at a resolution of 1.8 Å^{74, 75}. The ferrochelatase consists of two similar domains each with a four-parallel β -sheet flanked by α -helices. The active site is formed by protruding elements from both domains as a deep cleft. The structure of ferrochelatase in complex with the competitive inhibitor *N*-methyl mesoporphyrin IX and the artificial product Cu-mesoporphyrin IX have provided insight into the enzymatic mechanism of porphyrin metallation⁷⁵. These data support a chelation mechanism involving strain and distortion of the tetrapyrrole-based substrate, in which the pyrrole rings B, C and D are fixed by ferrochelatase in a 'vice-like grip' while ring A is forced into a 35° tilted conformation. In this configuration, two protons can be removed from the pyrrole-nitrogens and the metal ion can enter the macrocyclic cavity via the tilted ring A.

3. BIOSYNTHESIS OF LINEAR TETRAPYRROLES

3.1. Heme Oxygenases

Heme oxygenases catalyze the oxygen-dependent cleavage of the heme macrocycle with the release of Fe²⁺, CO and the linear tetrapyrrole biliverdin. They are not only essential for the bacterial iron metabolism and consequently pathogenesis, they also play important roles during oxidative stress responses, heme catabolism and for the biosynthesis of phytylbilins^{76–78}. Under iron limiting conditions, *P. aeruginosa* can use heme as its sole source of iron⁷⁹. In order to obtain the iron from heme, the heme macrocycle has to be cleaved by a heme oxygenase. *P. aeruginosa* has two potential heme oxygenases. The first heme oxygenase PtgA, originally identified as an iron-starvation protein⁸⁰, plays a key role in the iron metabolism of the bacterium. Its expression is under the

control of the transcriptional regulator Fur and thus *pigA* is only expressed when iron is limiting¹³. Interestingly, this protein converts heme to the unusual biliverdin isomers (BV) IX β and IX δ ^{13, 81} (see Figure 7). Why *P. aeruginosa* produces these particular BV isomers, their biological function is currently unknown. More recently, another potential heme oxygenase was identified in *P. aeruginosa*⁸². The gene for this heme oxygenase forms an operon together with the gene encoding a bacterial phytochrome (*bphP*) and was therefore designated *bphO*⁸². Heme oxygenase function of *P. aeruginosa* BphO was proven by its green color of *E. coli* cells expressing *bphO* due to the formation of BV from heme⁸³. The occurrence of *bphO* together in an operon with *bphP* suggests that BphO produces the prosthetic group for this plant-like photoreceptor⁸². Currently, the exact function of this phytochrome-based sensor-kinase is unknown.

Table 1 summarizes all heme and open chain tetrapyrrole biosynthetic enzymes and their corresponding gene products.

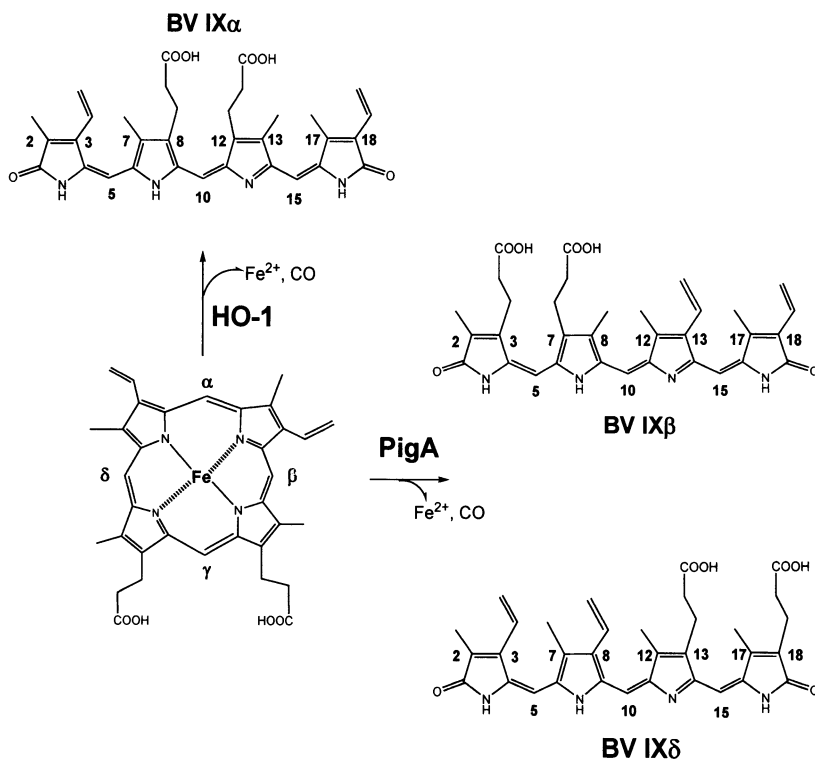


Figure 7. Reaction catalyzed by known heme oxygenases: HO-1 found in mammals, plants, cyanobacteria and possibly *P. aeruginosa* targets the α -meso carbon bridge of the porphyrin macrocycle whereas PigA from *P. aeruginosa* targets the β - and δ -meso bridge; both reactions leading to biliverdin, iron and carbon monoxide.

Table 1. Summary of all genes encoding heme and linear tetrapyrrole biosynthetic enzymes and their gene products.

Gene	Gene product	<i>P. aeruginosa</i>			
		PAOI	<i>P. fluorescens</i>	<i>P. putida</i> KT2440	<i>P. syringae</i>
<i>hemA</i>	Glutamyl-tRNA reductase	PA4666 ^a	✓ ^b	✓	✓
<i>hemL</i>	Glutamate-1-semialdehyde 2,1-aminomutase	PA3977	✓	✓	✓
<i>hemB</i>	Porphobilinogen synthase	PA5243	✓	✓	✓
<i>hemC</i>	Porphobilinogen deaminase	PA5260	✓	✓	✓
<i>hemD</i>	Uroporphyrinogen III synthase	PA5259	✓	✓	✓
<i>hemE</i>	Uroporphyrinogen III decarboxylase	PA5034	✓	✓	✓
<i>Unknown gene^c</i>	Protoporphyrinogen oxidase	?	?	?	?
<i>hemG</i>	Part of protoporphyrinogen oxidase	n.d.	n.d.	n.d.	n.d.
<i>hemY</i>	Protoporphyrinogen oxidase	n.d.	n.d.	n.d.	n.d.
<i>hemF</i>	Oxygen-dependent coproporphyrinogen oxidase	PA0024	✓	✓	✓
<i>hemN</i>	Oxygen-independent coproporphyrinogen oxidase	PA1546	n.d.	✓	✓
<i>hemH</i>	Ferrochelatase	PA4655	✓	✓	✓
<i>hemO/pigA</i>	Heme oxygenase	PA0672	✓	✓	✓
<i>bphO</i>	Heme oxygenase	PA4116	✓	✓	✓

^aLocus number according to the *P. aeruginosa* sequencing project; data available under <http://www.pseudomonas.com>

^bGene present according to BLAST searches; n.d. not detected.

^cPseudomonads have no obvious homologue to known bacterial Protox genes; therefore, an unknown locus might encode such a protein; see text for details; ?, unknown.

4. SIROHEME

Siroheme is a prosthetic group associated with the six electron reduction processes of sulphite reductases and the majority of assimilatory nitrite reductases^{84, 85}. In this respect, siroheme plays an important role in the processing of the bulk of 'organic' nitrogen and sulphur in biological systems. In molecular terms, siroheme can be described as an iron-containing isobacteriochlorin

and is the simplest of all the modified tetrapyrroles found in nature, maintaining the eight carboxylic acid side chains that are also found on uroporphyrinogen III (Figure 1). This relatively simple structure has led some to suggest that siroheme may have had an evolutionary ancient origin, as the isobacteriochlorin structure is well suited to the anaerobic environment of an ancient world⁸⁶. Within sulphite and nitrite reductases, siroheme is covalently linked to an Fe-S center and assists in the delivery of electrons to the substrate. The detailed structural information gained from high resolution X-ray data of the *E. coli* sulphite reductase reveals the extensive ruffled conformation of the macrocycle at the active site of the enzyme and suggests that the carboxylic acid side chains may also play an important role in the catalytic process^{85, 86}.

4.1. Biosynthesis of Siroheme

Despite the wealth of data available on the structure and function of sulphite and nitrite reductases, it is only relatively recently that any advance has been made on understanding how siroheme is made in biological systems^{87, 88}. The biosynthesis of siroheme branches from the central tetrapyrrole pathway at uroporphyrinogen III (Figure 1)⁴⁷. While decarboxylation of uroporphyrinogen III mediates the route toward heme, methylation of uroporphyrinogen III at positions 2 and 7 yields an intermediate called precorrin-2 (dihydrosirohydrochlorin), which is also an intermediate for the biosynthesis of cobalamin and heme *d*₁ (Figure 1). This methylation requires two S-adenosyl-L-methionine molecules as the methyl donors for this transformation. Precorrin-2 is then oxidized by the removal of two protons and two electrons to yield sirohydrochlorin in a process that is NAD-dependent. Finally, ferrochelation into the macrocyclic core produces siroheme. In all organisms studied to date, siroheme is synthesized by methylation, dehydrogenation and ferrochelation of uroporphyrinogen III, although the number of enzymes required for this transformation varies considerably. In *Bacilli*, there are separate enzymes for each of these steps², that is, a separate methyltransferase, dehydrogenase and chelatase, whereas in yeast a methyltransferase and a bifunctional dehydrogenase/chelatase are employed^{89, 90}. However, in enteric bacteria⁸⁸ and also in *P. aeruginosa*, the transformation of uroporphyrinogen III into siroheme is accomplished by the action of a single enzyme, CysG, which houses methyltransferase, dehydrogenase and chelatase activities within the ~460 amino acids of its primary structure. CysG would appear to represent a fusion between a uroporphyrinogen III methyltransferase and a bifunctional dehydrogenase/ferrochelatase⁸⁷.

The *cysG* gene in *P. aeruginosa* is not found as part of an operon associated with sulphite or nitrite reduction. Instead, it is found in relative isolation adjacent to the seryl-tRNA synthetase gene. Interestingly, a separate uroporphyrinogen III methyltransferase gene (*cobA*) is found within a group

of genes associated with the assimilatory nitrite reductase. However, this gene would not appear to be in association with any known genes capable of converting precorrin-2 into siroheme. The relationship between the *cobA* found in this region of the chromosome, the *cysG*, the *cobA*-type of gene found in the heme d_1 biosynthetic operon (*nirE*) and the *cobA* found in the main cobalamin operon is not understood. However, the high level of enzyme multiplicity suggests that this is a key regulatory enzyme that may be susceptible to various levels of feedback inhibition.

5. BIOSYNTHESIS OF VITAMIN B₁₂

In contrast to siroheme, which represents the simplest of the modified tetrapyrroles, vitamin B₁₂ (cobalamin) is the most complex member of the modified-tetrapyrrole family (Figure 1)^{1, 2, 91}. Indeed, vitamin B₁₂ is one of the most complex small molecules made in Nature, requiring around 30 enzymes for its complete *de novo* synthesis, which is typically underpinned by a genetic investment representing approximately 1% of a bacterial genome⁹². Despite this huge biosynthetic effort, the biologically active forms of vitamin B₁₂, adenosylcobalamin and methylcobalamin, are required for comparatively few enzymes. Cobalamin-requiring enzymes include those involved in rearrangement (e.g., methylmalonyl CoA mutase; ethanolamine ammonia lyase) and methylation processes (e.g., methionine synthase). Key to the action of cobalamin is the centrally chelated cobalt ion and the unique nature of the cobalt–carbon bond, which mediates much of the chemistry associated with cobalamin-dependent reactions. More detailed accounts of the enzymology associated with vitamin B₁₂-dependent reactions can be found elsewhere (see e.g., ref. [93]).

The intricate and complex chemical structure of vitamin B₁₂ is reflected in an equally complex and intricate biosynthetic pathway. The situation is further complicated by the appearance in Nature of two separate pathways, representing aerobic and anaerobic routes^{89, 94}. The aerobic pathway requires molecular oxygen as a substrate while the anaerobic pathway can operate effectively under both aerobic and anaerobic conditions. An important difference between the pathways relates to the timing of cobalt insertion, since the metal is inserted at an early stage in the anaerobic pathway but is chelated at a much later stage in the aerobic route by distinctly different cobalt chelatascs. As with heme d_1 and siroheme synthesis, cobalamin synthesis branches from the main tetrapyrrole assembly pathway at the uroporphyrinogen III junction by methylation at positions 2 and 7 of the macrocycle to give precorrin-2. This intermediate is common to both the aerobic and anaerobic routes for cobalamin biosynthesis and represents the point at which the two pathways diverge, before they rejoin again at the level of an intermediate called adenosylcobyrinic acid.

In this chapter, we shall concentrate on the aerobic pathway, since this is the pathway that appears to operate in many pseudomonads. The aerobic pathway is also the better characterized of the pathways since its step-by-step synthesis has been largely elucidated. Although the aerobic pathway was elucidated in the organism *Pseudomonas denitrificans*, the taxonomical description of this organism is vague and it is clear from the reported gene sequences and the presence of a 'Shemin route' for aminolevulinic acid synthesis that this organism is probably a member of the α -subclass of the *Proteobacteria*, most likely either a *Rhizobium* or an *Agrobacterium*. However, with some interesting variations, a similar aerobic pathway for cobalamin biosynthesis appears in *P. aeruginosa*, so we shall describe cobalamin biosynthesis in relation to this characterized aerobic pathway.

5.1. Aerobic Pathway of Cobalamin Biosynthesis

The transformation of uroporphyrinogen III into cobalamin requires the addition of eight methyl groups, ring contraction with the loss of the methylated meso C-20 carbon, decarboxylation, six amidations, cobalt insertion, aminopropanol attachment, adenosylation and the attachment of the lower nucleotide (α -ribazole). The naming of the intermediates synthesized along the pathway reflects both trivial names given to a few intermediates whose identity had been determined and a more systematic approach that was developed for the large number of intermediates that had yet to be elucidated. Thus, the name precorrin-*n* reflects intermediates prior to the synthesis of the main corrin component of vitamin B₁₂, where *n* reflects the number of methyl groups added to the macrocyclic template during its *de novo* construction.

For aerobic cobalamin biosynthesis⁹⁴⁻⁹⁶, the transformation of uroporphyrinogen III into cobalamin is initiated by the methylation of uroporphyrinogen III at positions 2 and 7 by the enzyme uroporphyrinogen III methyltransferase, which is encoded by *cobA* (Figure 8). In general, the genes for the aerobic cobalamin pathway are given the prefix *cob* for *cobalamin* biosynthesis. In contrast, the genes for the anaerobic pathway are given the prefix *cbi* for *cobinamide* biosynthesis, while the genes for the transformation of cobinamide into cobalamin are given the prefix *cob*. The *bis*-methylation of uroporphyrinogen III yields precorrin-2 (Figure 8). The next enzyme in the aerobic pathway is another methyltransferase, CobI, which methylates at C-20 to give precorrin-3A (Figure 8). This methyl group and the C-20 position are lost subsequently during the ring contraction process. Precorrin-3A acts as the substrate for a monooxygenase called CobG, which is an Fe-S containing protein that most likely also houses a non-heme iron. This enzyme catalyzes the synthesis of a hydroxylactone derivative of precorrin-3A called precorrin-3B,

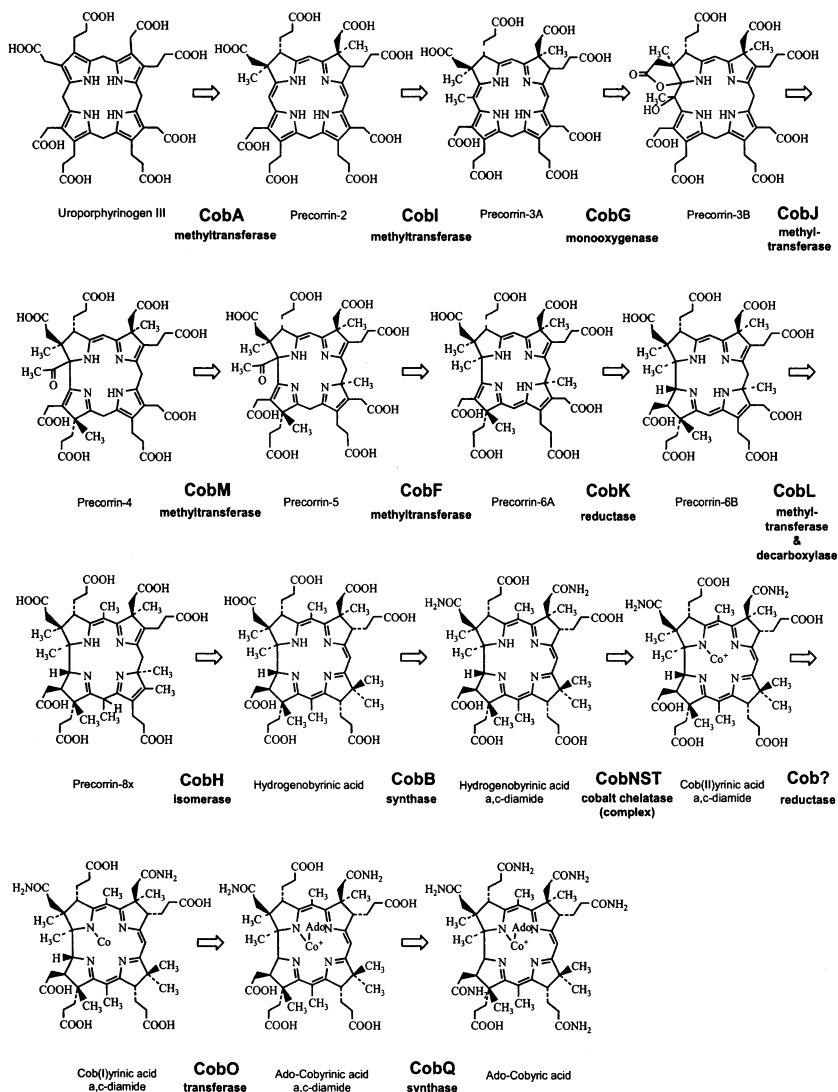


Figure 8. The step-by-step transformation of uroporphyrinogen III into adenosylcobyrinic acid is shown with the required enzymes. Although the enzyme associated with the reduction of the central cobalt ion has been described, no gene has yet been identified which encodes for this protein.

with the gamma lactone formed from the acetic acid side chain on ring A, and the hydroxy group on C-20 derived from molecular oxygen (Figure 8).

Precorrin-3B represents the spring-loaded substrate for the ring contraction process, which is catalyzed by the next enzyme CobJ. In fact, CobJ is another methyltransferase that methylates at C-17 (Figure 8). The methyltransferase

reaction promotes the ring contraction that leads to a carbon–carbon bond being formed between C-19 and C-1. This generates precorrin-4, which is further methylated by the next methyltransferase CobM to give precorrin-5 (Figure 8). CobM methylates the macrocycle at position C-11 although the methyl group is later moved by an isomerase to its final position on C-12. Precorrin-5 is converted into precorrin-6A with methylation at C-1 by CobF, a process that is concerted with the loss of the extruded methylated C-20 carbon fragment as acetic acid. During the ring contraction process, the overall oxidation state of the macrocycle is increased and the reduction of the intermediate back to the level of a hexahydroporphyrin is afforded by an enzyme called CobK, which requires NADPH as a cofactor, and converts precorrin-6A into precorrin-6B (Figure 8). The final two methyl groups are added to precorrin-6B by a bifunctional enzyme termed CobL, which methylates at positions 5 and 15 and also catalyzes the decarboxylation of the acetic acid side chain attached to C-12. It is likely that this enzyme first methylates at C-5 and that the methylation of C-15 is used to drive the decarboxylation of the acetic acid side chain. The product of this reaction is precorrin-8, which is finally converted into hydrogenobyrrinic acid by CobH, an isomerase that catalyzes the 1,5 sigmatropic rearrangement of the methyl group from C-11 to C-12 (Figure 8).

This stage in the synthesis of vitamin B₁₂ sees the end of the methylation reactions and focus is then centered on the amidations and metal chelation processes. Initially, hydrogenobyrrinic acid is amidated on the acetic acid side chains found on rings A and B by the enzyme CobQ, generating hydrogenobyrrinic acid a,c-diamide (Figure 8). This intermediate acts as the substrate for the cobaltochelatease, which in *P. denitrificans* is a large protein complex that requires three protein subunits, CobN, S and T. The chelation reaction requires ATP for the insertion of the central metal ion into the corrin ring, in a process that has some similarities with the insertion of magnesium during chlorophyll biosynthesis⁹⁷. The product of the reaction is cobyrinic acid a,c-diamide, which acts as the substrate for the adenosylation enzyme CobO. Initially, the cobalt ion in the corrin ring is first reduced to cobalt (I) by a flavin-dependent reductase. The cobalt (I) ion acts as a strong nucleophile and in the presence of CobO and ATP leads to the synthesis of adenosylcobyrinic acid a,c-diamide (Figure 8). The remaining four amidations of the acid side chains found at C-3, C-8, C-13 and C-18 are catalyzed by CobQ and yield adenosylcobyrinic acid (Figure 8).

With the synthesis of the main corrin ring now complete, the final steps in cobalamin biosynthesis are concerned with the synthesis and attachment of the lower axial cobalt ligand, which is housed within a modified nucleotide called α -ribazole. The lower axial ligand is attached to the main corrin framework by an aminopropanol arm. This aminopropanol is synthesized from L-threonine, which is initially phosphorylated to give L-threonine-O-3-phosphate and subsequently decarboxylated to give aminopropanol-O-2-phosphate (Figure 9).

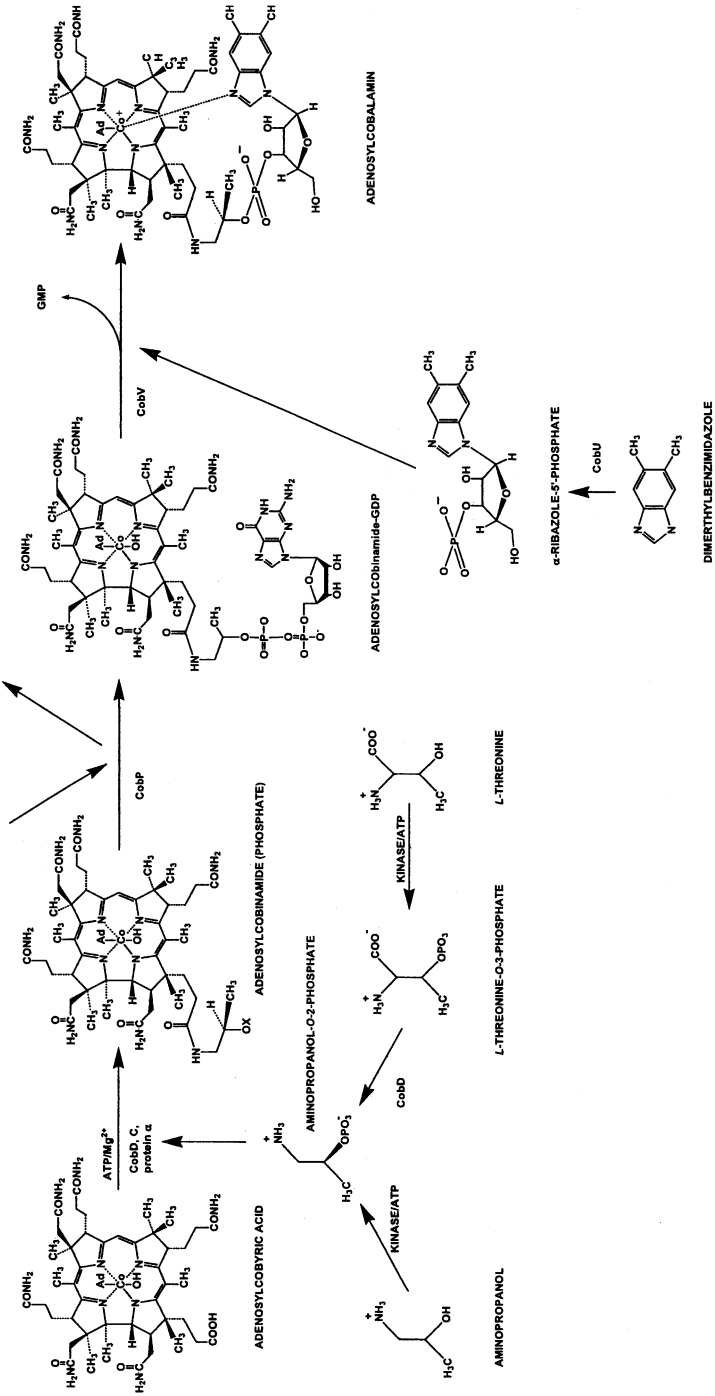


Figure 9. The step-by-step transformation of adenosylcobyrinic acid into adenosylcobalamin.

The latter reaction is catalyzed by CobC, which is thought to be a pyridoxal-requiring enzyme. The attachment of the aminopropanol-O-2-phosphate to adenosylcobyric acid is mediated by a complex formed between CobD and protein a. This generates adenosylcobinamide phosphate (Figure 9), which acts as the substrate for CobP, an enzyme that attaches GMP to the aminopropanol phosphate tail to give adenosyl-GDP-cobinamide. The terminal steps of cobalamin synthesis require the construction of the α -ribazole and its attachment to cobinamide. The α -ribazole is constructed by the transfer of the phosphoribosyl group of nicotinate mononucleotide to the modified base dimethylbenzimidazole in a reaction that is catalyzed by CobU (Figure 9). Finally, the α -ribazole is attached to the adenosyl-GDP-cobinamide with the release of GMP by the action of CobV to generate adenosylcobalamin (Figure 9).

The sequence of the *P. aeruginosa* genome allows the genetic organization of the cobalamin biosynthetic genes to be viewed and it suggests that some unique biochemistry is taking place during cobalamin metabolism in this organism. The *cob* genes are contained within three main loci (Figure 10) although little is known about their control and regulation. Of particular significance is the presence of *cobG* and *cobN*, genes that encode for the precorrin-3a monooxygenase and the cobaltochelatease, respectively. Both these enzymes can be thought of as genetic hallmarks of the aerobic pathway. Although the cobalamin biosynthetic pathway has not been investigated in *P. aeruginosa*, this evidence strongly suggests that an oxygen-dependent (aerobic) pathway operates in this organism. However, a closer scrutiny of the genome reveals some oddities as a number of the aerobic enzymes would appear to be missing. There is no CobF (the C1 methyltransferase, a gene that has no homologue in the anaerobic pathway), and no CobS and T, the other components of the aerobic cobaltochelatease (Figure 10). Moreover, the operons in *P. aeruginosa* also contain genes that

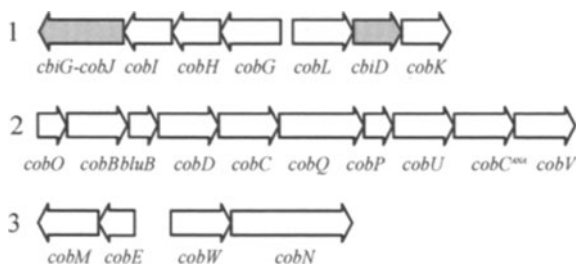


Figure 10. Organization of the genes for cobalamin biosynthesis in *P. aeruginosa*. The cobalamin biosynthetic genes are found in three major operons. Although they contain genes that have a high degree of similarity with genes associated with the 'aerobic' pathway, they also contain a few genes with a high degree of similarity with genes found in the anaerobic pathway, for example, *cbiD*, *cbiG* and *cobC^{ana}*.

encode two proteins, CbiD and CbiG (Figure 10), which had previously been only found associated with anaerobic pathway producers⁸⁹. Indeed, CbiG is found fused onto CobJ, making one large protein. Moreover, we have investigated the ability of *P. aeruginosa* to biosynthesize cobalamin under both aerobic and anaerobic conditions, and believe that *P. aeruginosa* has evolved a pathway that can cope with rapid changes in the level of available oxygen. There are a number of interesting questions that still need to be addressed for cobalamin biosynthesis in *P. aeruginosa*, including how the bacterium is able to make cobalamin in the absence of molecular oxygen, how it methylates at C-1 in the absence of CobF, and how its cobaltochelatase is able to function without the S and T subunits.

6. HEME d_1

6.1. Heme d_1 Containing Enzymes in *P. aeruginosa*

Pseudomonas aeruginosa contains a dissimilatory nitrite reductase, which is known as cytochrome cd_1 ¹⁰. As the name implies, this enzyme contains a C-type heme and a highly unusual modified tetrapyrrole called heme d_1 . In fact heme d_1 is not a porphyrin at all, but a dioxoisobacteriochlorin that bears more similarity to siroheme than to heme⁹⁹.

The native enzyme is a homodimer with a subunit mass of around 60 kDa¹⁰. The heme c and heme d_1 are both located in each subunit. The enzyme is involved in the reduction of nitrite to NO and catalyzes the oxidation of cytochrome $c551$. Although the heme c prosthetic group is covalently attached to the protein, the heme d_1 group is non-covalently bound. Indeed, this group can be removed and reintroduced to restore fully active enzyme. Moreover, chemically synthesized heme d_1 can also be introduced into *apo* enzyme to afford fully active enzyme. Such studies helped to confirm the structure of heme d_1 ¹⁰⁰.

The structure of cytochrome cd_1 was first described for the enzyme from *Paracoccus denitrificans*, which revealed the enzyme to consist of two major domains¹⁰¹. The N-terminus of the protein (amino acids 1–134) houses the heme c domain and is largely α -helical in content, while the C-terminus of the protein (amino acids 135–567) houses the heme d_1 domain in a β -propellor structure containing eight blades.

6.2. Biosynthesis of Heme d_1

Although significant progress has been made on the role and function of cytochrome cd_1 and on the contribution of heme d_1 toward catalysis, progress of the biosynthesis of this highly unusual tetrapyrrole-derived prosthetic group

has remained static and little is known how this molecule is biosynthesized. Genetic studies in organisms such as *P. aeruginosa* have at least allowed some or all of the genes associated with the biosynthesis of heme d_1 to be identified. The genes for heme d_1 biosynthesis are thought to be located within the main dissimilatory nitrite reductase operon¹⁰², which includes *nirSMCFDLGHJEN*. Out of these, insertional mutation and complementation analysis suggests that *nirFDLGHJE* are necessary for the biosynthesis of heme d_1 . Sequence similarity indicates that NirE is a uroporphyrinogen III methyltransferase, consistent with the view that heme d_1 is derived from the precorrin-2 branch of the tetrapyrrole biosynthetic pathway. NirJ has similarity with NirD, -L, -G and -H, suggesting some kind of gene duplication and evolutionary relationship between these proteins. Moreover, NirJ is thought to belong to the radical SAM class of enzymes, suggesting that the biosynthesis of heme d_1 may proceed via a mechanism involving a radical species⁶².

A comparison of the structure of heme d_1 with uroporphyrinogen III indicates that the transformation involves some unusual chemistry (Figure 1). Heme d_1 contains two methyl groups at C-2 and C-7, which are consistent with a biosynthesis via precorrin-2. Most striking, however, is the loss of the two propionic side chains at C-3 and C-8 and their replacement with oxo groups, a process that is made all the more difficult to explain since this synthesis is likely to occur under anaerobic conditions. Although it has been suggested that this process may proceed via hydroxylation of the propionate side chain followed by a reverse aldol condensation, the employment of a radical species via an internal lactone may prove to be an alluring possibility. Further modifications on the way to heme d_1 include the decarboxylation of the acetic side chains on C-12 and C-18, and the introduction of an acrylate substituent into the propionate side chain on C-17. The final modification for heme d_1 is the chelation of ferrous iron. The aforementioned steps including the decarboxylations, acrylate synthesis and ferrochelation all draw parallels with other known modifications associated with tetrapyrrole synthesis, yet no homologues of these enzymes, for example, for decarboxylation or ferrochelation, are found within the *nir* operon.

Although there is still much to be learnt about the biosynthesis of heme d_1 , there is equally much to be learnt about how the prosthetic group, once synthesized, is transported and inserted into the *apo* enzyme, which is located within the periplasm. Thus, logistical issues as well as the biosynthetic route for the synthesis of heme d_1 still need to be addressed.

7. REGULATION OF HEME BIOSYNTHESIS IN *P. AERUGINOSA*

The biosynthesis of heme is strictly controlled in most bacteria including *P. aeruginosa*. One important reason is that the accumulation of free heme

as well as some of the biosynthetic intermediates of the pathway are toxic to the cells¹⁰³. Moreover, the demand for heme in *P. aeruginosa* changes drastically in dependence of the employed mode of energy generation. Physiological experiments in the 1970s revealed that heme levels increase under anaerobic conditions up to 20-fold in *P. aeruginosa*. These investigations suggested oxygen-tension, nitrate availability and the supplied carbon source as direct or indirect signals for the regulation of heme biosynthesis^{11, 104, 105}. Heme itself is also a strong regulator of its own biosynthesis. Experiments using *E. coli* showed a 20-fold increase of heme production when heme binding proteins like hemoglobin, cytochromes or catalases were recombinantly overproduced^{106, 107}.

Two steps of the heme biosynthesis pathway were identified as key regulatory points^{11, 108}. The first regulatory target is the initial step of heme biosynthesis catalyzed by glutamyl-tRNA reductase (HemA). The rate limiting character of this step was convincingly documented by ALA-feeding experiments. Increased heme formation in combination with the accumulation and excretion of coproporphyrin III was observed¹⁰⁸. Therefore, the second obvious regulatory point of heme biosynthesis is the formation of protoporphyrinogen IX from coproporphyrinogen III catalyzed by various coproporphyrinogen III oxidases named HemF, HemN and HemZ.

7.1. Regulation of the *hemA* Gene

Transcriptional regulation of *P. aeruginosa hemA* in response to changing environmental conditions was initially studied by quantification of formed mRNA. The *hemA* mRNA levels in *P. aeruginosa* increase under anaerobic denitrifying conditions compared to aerobic growth. They are very low under fermentative conditions where no heme containing cytochromes are required for respiration²⁰. Two transcriptional start sites were found upstream of *hemA*. Both transcripts are present during both aerobic and anaerobic growth conditions. A *P. aeruginosa* mutant with a defect in the oxygen regulator Anr (an *E. coli* Fnr homologue) exhibited similar overall increased *hemA* mRNA levels as the wild-type under anaerobic denitrifying conditions. However, the distribution of *hemA* mRNA from both promoters differed significantly²⁰. Regulation of *hemA* promoter 1 was further studied using reporter gene fusion. A moderate 2.8-fold anaerobic induction of transcription from the *hemA* promoter 1 was observed. This anaerobic induction was dependent on the regulatory proteins Anr, Dnr, NarL and Integration Host Factor (IHF)¹⁰⁹. Anr- and Dnr induce the *hemA* promoter cooperatively via a half site motif for Anr/Dnr binding. The regulatory proteins Anr and Dnr have been previously reported to form a regulatory cascade for the induction of denitrification genes under anaerobic conditions. During this cascade, Anr induces expression of *dnr*. Dnr is presumed to detect

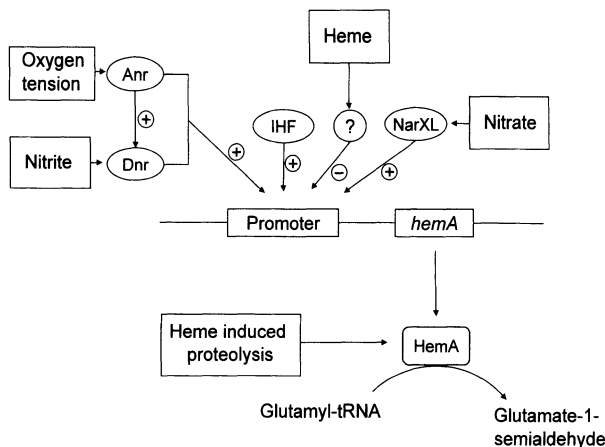


Figure 11. Overview of the regulation of the first tetrapyrrole biosynthesis step in *P. aeruginosa*. See text for details.

nitrite or nitrous oxide and in turn induces transcription of various target genes^{110, 111}. In contrast to the regulation of *hemA* transcription in *E. coli* and *Salmonella typhimurium*, the anaerobic induction of *P. aeruginosa hemA* was found dependent on the presence of nitrate. This control was mediated by the two component regulatory system encoded by *narXL* (Figure 11)¹⁰⁹.

Studies with the HemA protein in *E. coli* unraveled that the major control of HemA formation takes place at the protein level^{112–114}. Heme has no obvious inhibitory effect on the enzymatic activity of the purified HemA protein¹¹⁵; however, the glutamyl-tRNA reductase activity is significantly reduced when heme is added to cell free extracts of *E. coli*¹¹⁶. This implies, that additional factors in combination with heme are required for the regulation of HemA activity. Elliott and coworkers first discovered a striking difference between *hemA-lacZ* expression and enzymatic activity of glutamyl-tRNA reductase in various *S. typhimurium* heme deficient mutants¹¹². While *hemA-lacZ* expression increased only 1.5–2-fold in heme reduced *hemB* or *hemL* mutants, the enzymatic activity of glutamyl-tRNA reductase increased 20–25-fold proportional to the parallel increase of HemA protein. These data obtained for *E. coli* and *S. typhimurium* are almost identical to the observed moderate 2.8-fold increase of *hemA-lacZ* expression in anaerobically grown *P. aeruginosa* compared to the parallel 20-fold increase of heme levels¹⁰⁹. Pulse-chase experiments of HemA turnover in *hemL* mutants of *S. typhimurium* revealed a half-life of 20 min in the presence of ALA which increased to more than 300 min in heme limited cells¹¹³. Two ATP-dependent proteases Lon and ClpP as well as the ClpA chaperone were identified to be involved in HemA turnover. In an *E. coli lon clpP* double mutant, the HemA

protein showed a significantly increased half life. These results clearly demonstrated that cellular HemA concentration is regulated by conditional protein stability¹¹³. A HemA mutant protein with a positive charge introduced close to the N-terminus showed significantly increased stability. These results suggest that the N-terminal active site domain is the major target for proteolysis by the Lon and ClpP protease systems¹¹⁴.

7.2. Regulation of the *hemF* and *hemN* Genes

The second key regulatory point of heme biosynthesis, the conversion of coproporphyrinogen III is significantly regulated at the transcriptional level⁶³. In *P. aeruginosa* at least two different isoenzymes catalyze this step. Interestingly, the oxygen-dependent coproporphyrinogen III oxidase encoded by *hemF* and the oxygen-independent coproporphyrinogen III oxidase encoded by *hemN* share no similarity at the amino acid sequence level⁶³. Regulation of *hemF* and *hemN* was found not as simple as expected from their oxygen-related enzymatic functions. The 7–10-fold anaerobic induction was only expected in the case of the *hemN* gene encoding the oxygen-independent enzyme. Surprisingly, also *hemF* encoding the oxygen-dependent enzyme was found 20-fold induced under anaerobic conditions⁶³. Moreover, a *P. aeruginosa* *hemF* mutant showed significant reduced anaerobic growth indicating additional anaerobic functions for the HemF protein. The promoter regions of *hemF* and *hemN* both contain a putative Anr/Dnr binding motif 44.5 bp and

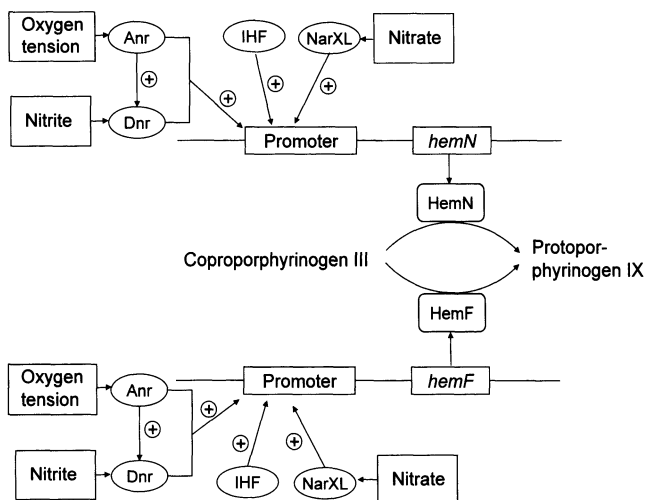


Figure 12. Model for the regulation of *hemF* and *hemN* encoding the oxygen-dependent and -independent coproporphyrinogen oxidases.

41.5 bp upstream of the transcriptional start site, respectively⁶³. Investigation of *P. aeruginosa* *hemF* and *hemN* transcription using *anr* or *dnr* mutant strains and co-expression of the *anr* and *dnr* genes *in trans* revealed dual action of Anr and Dnr during the anaerobic transcriptional induction process. Surprisingly, Anr is also essential for aerobic expression of *hemN*⁶³. Moreover, observed anaerobic *hemF* and *hemN* expression requires in parallel nitrate induction via the NarXL system and DNA-bending via IHF (Figure 12).

Only limited information is available about the regulation of the remaining heme biosynthesis steps in *P. aeruginosa*. Expression of *P. aeruginosa* *hemL* encoding glutamate-1-semialdehyde-2-1-aminomutase was found induced by oxygen stress and anaerobic denitrifying conditions²². Microarray experiments showed a 2-fold upregulation of the *hemB* gene encoding PBGS under anaerobic conditions.

8. SUMMARY AND CONCLUDING REMARKS

Crucial to the efficient deployment in bacteria of aerobic and anaerobic energy conserving systems is the role played by modified tetrapyrroles, whose imposing molecular framework is also able to mediate complex enzymatic reactions, regulatory processes and stress responses. *P. aeruginosa* is one of a select number of bacteria that have the ability to make five different types of modified tetrapyrroles, including various hemes, siroheme, cobalamin (vitamin B₁₂), heme *d*₁ and open chain compounds. The molecular design of these prosthetic groups reflects the fact that they are all derived from a common macrocyclic intermediate called uroporphyrinogen III, which is snipped, hammered and welded into the required final form by the enzymes within a large branched biosynthetic pathway. The simplest member of this tetrapyrrole-fraternity is siroheme, which requires six enzymes for its complete *de novo* synthesis, whereas the most complex family member (vitamin B₁₂) requires a total of 26 enzymes. In this chapter, we reviewed our current understanding of the step-by-step synthesis of this intricate and complicated branched pathway. Moreover, we outline how tetrapyrrole synthesis is controlled and regulated, especially in response to changes in cellular environment.

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REFERENCES

1. Martens, J.H., Barg, H., Warren, M.J., and Jahn, D., 2002, Microbial production of vitamin B12. *Appl. Microbiol. Biotechnol.*, 58:275–285.
2. Raux, E., Leech, H.K., Beck, R., Schubert, H.L., Santander, P.J., Roessner, C.A., Scott, A.I., Martens, J.H., Jahn, D., Thermes, C., Rambach, A., and Warren, M.J., 2003, Identification and functional analysis of enzymes required for precorrin-2 dehydrogenation and metal ion insertion in the biosynthesis of sirohaem and cobalamin in *Bacillus megaterium*. *Biochem. J.* 370:505–516.
3. Chang, C.K., 1994, Haem d1 and other haem cofactors from bacteria. *Ciba Found. Symp.*, 180:228–238; discussion 238–246.
4. O'Brian, M.R. and Thöny-Meyer, L., 2002, Biochemistry, regulation and genomics of haem biosynthesis in prokaryotes. *Adv. Microb. Physiol.*, 46:257–318.
5. Schmitt, M.P., 1997, Utilization of host iron sources by *Corynebacterium diphtheriae*: Identification of a gene whose product is homologous to eukaryotic heme oxygenases and is required for acquisition of iron from heme and hemoglobin. *J. Bacteriol.*, 179:838–845.
6. Letoffe, S., Nato, F., Goldberg, M.E., and Wandersman, C., 1999, Interactions of HasA, a bacterial haemophore, with haemoglobin and with its outer membrane receptor HasR. *Mol. Microbiol.*, 33:546–555.
7. Ogawa, K., Sun, J., Taketani, S., Nakajima, O., Nishitani, C., Sassa, S., Hayashi, N., Yamamoto, M., Shibahara, S., Fujita, H., and Igarashi, K., 2001, Heme mediates derepression of Maf recognition element through direct binding to transcription repressor Bach1. *EMBO J.*, 20:2835–2843.
8. Schmitt, M.P., 1999, Identification of a two-component signal transduction system from *Corynebacterium diphtheriae* that activates gene expression in response to the presence of heme and hemoglobin. *J. Bacteriol.*, 181:5330–5340.
9. Chen, J.J. and London, I.M., 1995, Regulation of protein synthesis by heme-regulated eIF-2 alpha kinase. *Trends Biochem. Sci.*, 20:105–108.
10. Zumft, W.G. 1997, Cell biology and molecular basis of denitrification. *Microbiol. Mol. Biol. Rev.*, 61:533–616.
11. Jacobs, N.J., Jacobs, J.M., and Morgan, H.E., 1972, Comparative effect of oxygen and nitrate on protoporphyrin and heme synthesis from δ -aminolevulinic acid in bacterial cultures. *J. Bacteriol.*, 112:1444–1445.
12. Cooper, M., Tavankar, G.R., and Williams, H.D. 2003, Regulation of expression of the cyanide-insensitive terminal oxidase in *Pseudomonas aeruginosa*. *Microbiology*, 149:1275–1284.
13. Ratliff, M., Zhu, W., Deshmukh, R., Wilks, A., and Stojiljkovic, I., 2001, Homologues of Neisserial Heme Oxygenase in Gram-Negative Bacteria: Degradation of heme by the product of the *pigA* gene of *Pseudomonas aeruginosa*. *J. Bacteriol.*, 183:6394–6403.
14. Frankenberg, N. and Lagarias, J.C., 2003, Biosynthesis and biological functions of bilins. In K.M. Kadish, K.M. Smith, and R. Guilard (eds), *The Porphyrin Handbook*, vol. 13 Elsevier Science, USA.
15. Shemin, D. and Russell, C.S., 1953, Delta-aminolevulinic acid, its role in the biosynthesis of porphyrins and purines. *J. Am. Chem. Soc.*, 75:4873–4875.
16. Beale, S.I. and Castelfranco, P.A., 1973, ¹⁴C incorporation from exogenous compounds into δ -aminolevulinic acid by greening cucumber cotyledons. *Biochem. Biophys. Res. Commun.*, 52:143–149.
17. Jahn, D., Verkamp, E., and Söll, D., 1992, Glutamyl-transfer RNA: A precursor of heme and chlorophyll biosynthesis. *Trends Biochem. Sci.*, 17:215–218.

18. Kikuchi, G., Kumar, A.M., Tamalge, P., and Shemin, D., 1958, The enzymatic synthesis of δ -aminolevulinic acid. *J. Biol. Chem.*, 233:1214–1219.
19. Gibson, K.D., Laver, W.G., and Neuberger, A., 1958, Initial steps in the biosynthesis of porphyrins. The formation of δ -aminolevulinic acid from glycine and succinyl-CoA by particles of chicken erythrocytes. *Biochem. J.*, 70:71–81.
20. Hungerer, C., Troup, B., Romling, U., and Jahn, D., 1995, Regulation of the *hemA* gene during 5-aminolevulinic acid formation in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 177:1435–1443.
21. Ilag, L.L. and Jahn, D., 1992, Activity and spectroscopic properties of the *Escherichia coli* glutamate 1-semialdehyde aminotransferase and the putative active site mutant K265R. *Biochemistry*, 31:7143–7151.
22. Hungerer, C., Troup, B., Romling, U., and Jahn, D., 1995, Cloning, mapping and characterization of the *Pseudomonas aeruginosa hemL* gene. *Mol. Gen. Genet.*, 248:375–380.
23. Weinstein, J.D. and Beale, S.I., 1983, Separate physiological roles and subcellular compartments for two tetrapyrrole biosynthetic pathways in *Euglena gracilis*. *J. Biol. Chem.*, 258:6799–6807.
24. O'Neill, G.P. and Soll, D., 1990, Transfer RNA and the formation of the heme and chlorophyll precursor, 5-aminolevulinic acid. *Biofactors*, 2:227–235.
25. Moser, J., Lorenz, S., Hubschwerlen, C., Rompf, A., and Jahn, D., 1999, *Methanopyrus kandleri* Glutamyl-tRNA Reductase. *J. Biol. Chem.*, 274:30679–30685.
26. Moser, J., Schubert, W.D., Beier, V., Bringemeier, I., Jahn, D., and Heinz, D.W., 2001, V-shaped structure of glutamyl-tRNA reductase, the first enzyme of tRNA-dependent tetrapyrrole biosynthesis. *EMBO J.*, 20:6583–6590.
27. Schubert, W.-D., Moser, J., Schauer, S., Heinz, D.W., and Jahn, D., 2002, Structure and function of glutamyl-tRNA reductase, the first enzyme of tetrapyrrole biosynthesis in plants and prokaryotes. *Photosynth. Res.*, 74:205–215.
28. Schauer, S., Chaturvedi, S., Randau, L., Moser, J., Kitabatake, M., Lorenz, S., Verkamp, E., Schubert, W.D., Nakayashiki, T., Murai, M., Wall, K., Thomann, H.U., Heinz, D.W., Inokuchi, H., Söll, D., and Jahn, D., 2002, *Escherichia coli* glutamyl-tRNA reductase. Trapping the thioester intermediate. *J. Biol. Chem.*, 277:48657–48663.
29. Smith, M.A., Kannangara, C.G., Grimm, B., and von Wettstein, D., 1991, Characterization of glutamate-1-semialdehyde aminotransferase of *Synechococcus*. Steady-state kinetic analysis. *Eur. J. Biochem.*, 202:749–757.
30. Smith, M.A., Grimm, B., Kannangara, C.G., and von Wettstein, D., 1991, Spectral kinetics of glutamate-1-semialdehyde aminomutase of *Synechococcus*. *Proc. Natl. Acad. Sci. USA*, 88:9775–9779.
31. Friedmann, H.C., Duban, M.E., Valasinas, A., and Frydman, B., 1992, The enantioselective participation of (S)- and (R)-diaminovaleric acids in the formation of delta-aminolevulinic acid in cyanobacteria. *Biochem. Biophys. Res. Commun.*, 185:60–68.
32. Grimm, B., Smith, M.A., and von Wettstein, D., 1992, The role of Lys272 in the pyridoxal 5-phosphate active site of *Synechococcus* glutamate-1-semialdehyde aminotransferase. *Eur. J. Biochem.*, 206:579–585.
33. Hennig, M., Grimm, B., Contestabile, R., John, R.A., and Jansonius, J.N., 1997, Crystal structure of glutamate-1-semialdehyde aminomutase: An alpha2-dimeric vitamin B6-dependent enzyme with asymmetry in structure and active site reactivity. *Proc. Natl. Acad. Sci. USA*, 94:4866–4871.
34. Contestabile, R., Angelaccio, S., Maytum, R., Bossa, F., and John, R.A., 2000, The contribution of a conformationally mobile, active site loop to the reaction catalyzed by glutamate semialdehyde aminomutase. *J. Biol. Chem.*, 275:3879–3886.

35. Shoolingin-Jordan, P.M., Spencer, P., Sarwar, M., Erskine, P.E., Cheung, K.M., Cooper, J.B., and Norton, E.B., 2002, 5-Aminolaevulinic acid dehydratase: Metals, mutants and mechanism. *Biochem. Soc. Trans.*, 30:584–590.
36. Frankenberg, N., Kittel, T., Hungerer, C., Romling, U., and Jahn, D., 1998, Cloning, mapping and functional characterization of the *hemB* gene of *Pseudomonas aeruginosa*, which encodes a magnesium-dependent 5-aminolevulinic acid dehydratase. *Mol. Gen. Genet.*, 257:485–489.
37. Frankenberg, N., Heinz, D.W., and Jahn, D., 1999, Production, purification, and characterization of a Mg^{2+} -responsive porphobilinogen synthase from *Pseudomonas aeruginosa*. *Biochemistry*, 38:13968–13975.
38. Erskine, P.T., Senior, N., Awan, S., Lambert, R., Lewis, G., Tickle, I.J., Sarwar, M., Spencer, P., Thomas, P., Warren, M.J., Shoolingin-Jordan, P.M., Wood, S.P., and Cooper, J.B., 1997, X-ray structure of 5-aminolaevulinic acid dehydratase, a hybrid aldolase. *Nat. Struct. Biol.*, 4:1025–1031.
39. Erskine, P.T., Norton, E., Cooper, J.B., Lambert, R., Coker, A., Lewis, G., Spencer, P., Sarwar, M., Wood, S.P., Warren, M.J., and Shoolingin-Jordan, P.M., 1999, X-ray structure of 5-aminolevulinic acid dehydratase from *Escherichia coli* complexed with the inhibitor levulinic acid at 2.0 Å resolution. *Biochemistry*, 38:4266–4276.
40. Frankenberg, N., Erskine, P.T., Cooper, J.B., Shoolingin-Jordan, P.M., Jahn, D., and Heinz, D.W., 1999, High resolution crystal structure of a Mg^{2+} -dependent porphobilinogen synthase. *J. Mol. Biol.*, 289:591–602.
41. Frere, F., Schubert, W.D., Stauffer, F., Frankenberg, N., Neier, R., Jahn, D., and Heinz, D.W., 2002, Structure of porphobilinogen synthase from *Pseudomonas aeruginosa* in complex with 5-fluorolevulinic acid suggests a double Schiff base mechanism. *J. Mol. Biol.*, 320:237–247.
42. Frankenberg, N., Jahn, D., and Jaffe, E.K., 1999, *Pseudomonas aeruginosa* contains a novel type V porphobilinogen synthase with no required catalytic metal ions. *Biochemistry*, 38:13976–13982.
43. Jaffe, E.K., 2003, An unusual phylogenetic variation in the metal ion binding sites of porphobilinogen synthase. *Chem. Biol.*, 10:25–34.
44. Jordan, P.M. and Warren, M.J., 1987, Evidence for a dipyrromethane cofactor at the catalytic site of *E. coli* porphobilinogen deaminase. *FEBS Lett.*, 225:87–92.
45. Jordan, P.M., 1994, Highlights in haem biosynthesis. *Curr. Opin. Struct. Biol.*, 4:902–911.
46. Hadener, A., Matzinger, P.K., Battersby, A.R., McSweeney, S., Thompson, A.W., Hammersley, A.P., Harrop, S.J., Cassetta, A., Deacon, A., Hunter, W.N., Nieh, Y.P., Raftery, J., Hunter, N., and Helliwell, J.R., 1999, Determination of the structure of seleno-methionine-labelled hydroxymethylbilane synthase in its active form by multi-wavelength anomalous dispersion. *Acta Crystallogr. D Biol. Crystallogr.*, 55(Pt 3):631–643.
47. Warren, M.J. and Scott, A.I., 1990, Tetrapyrrole assembly and modification into the ligands of biologically functional cofactors. *Trends Biochem. Sci.*, 15:486–491.
48. Helliwell, J.R., Nieh, Y.P., Habash, J., Faulder, P.F., Raftery, J., Cianci, M., Wulff, M., and Hadener, A., 2003, Time-resolved and static-ensemble structural chemistry of hydroxymethylbilane synthase. *Faraday Discuss.*, 122:131–144; discussion 171–190.
49. Mohr, C.D., Sonstebj, S.K., and Deretic, V., 1994, The *Pseudomonas aeruginosa* homologs of *hemC* and *hemD* are linked to the gene encoding the regulator of mucoidy AlgR. *Mol. Gen. Genet.*, 242:177–184.
50. Mathews, M.A., Schubert, H.L., Whitby, F.G., Alexander, K.J., Schadick, K., Bergonia, H.A., Phillips, J.D., and Hill, C.P., 2001, Crystal structure of human uroporphyrinogen III synthase. *EMBO J.*, 20:5832–5839.
51. Schubert, H.L., Raux, E., Mathews, M.A., Phillips, J.D., Wilson, K.S., Hill, C.P., and Warren, M.J., 2002, Structural diversity in metal ion chelation and the structure of uroporphyrinogen III synthase. *Biochem. Soc. Trans.*, 30:595–600.

52. Akhtar, M., 1991, Mechanism and stereochemistry of the enzymes involved in the conversion of uroporphyrinogen III into haem. In P.M. Jordan, (ed.), *Biosynthesis of Tetrapyrroles*, pp. 67–99, Elsevier Science Publishers, Amsterdam.
53. Martins, B.M., Grimm, B., Mock, H.P., Huber, R., and Messerschmidt, A., 2001, Crystal structure and substrate binding modeling of the uroporphyrinogen-III decarboxylase from *Nicotiana tabacum*. Implications for the catalytic mechanism. *J. Biol. Chem.*, 276:44108–44116.
54. Whitby, F.G., Phillips, J.D., Kushner, J.P., and Hill, C.P., 1998, Crystal structure of human uroporphyrinogen decarboxylase. *EMBO J.*, 17:2463–2471.
55. de Verneuil, H., Sassa, S., and Kappas, A., 1983, Purification and properties of uroporphyrinogen decarboxylase from human erythrocytes. A single enzyme catalyzing the four sequential decarboxylations of uroporphyrinogens I and III. *J. Biol. Chem.*, 258:2454–2460.
56. Straka, J.G. and Kushner, J.P., 1983, Purification and characterization of bovine hepatic uroporphyrinogen decarboxylase. *Biochemistry*, 22:4664–4672.
57. Dailey, H.A., 2002, Terminal steps of haem biosynthesis. *Biochem. Soc. Trans.*, 30:590–595.
58. Colloc'h, N., Mornon, J.P., and Camadro, J.M., 2002, Towards a new T-fold protein?: The coproporphyrinogen III oxidase sequence matches many structural features from urate oxidase. *FEBS Lett.*, 526:5–10.
59. Medlock, A.E. and Dailey, H.A., 1996, Human coproporphyrinogen oxidase is not a metallo-protein. *J. Biol. Chem.*, 271:32507–32510.
60. Homuth, G., Rompf, A., Schumann, W., and Jahn, D., 1999, Transcriptional control of *Bacillus subtilis* *hemN* and *hemZ*. *J. Bacteriol.*, 181:5922–5929.
61. Layer, G., Verfurth, K., Mahlitz, E., and Jahn, D., 2002, Oxygen-independent coproporphyrinogen-III oxidase HemN from *Escherichia coli*. *J. Biol. Chem.*, 277:34136–34142.
62. Sofia, H.J., Chen, G., Hetzler, B.G., Reyes-Spindola, J.F., and Miller, N.E., 2001, Radical SAM, a novel protein superfamily linking unresolved steps in familiar biosynthetic pathways with radical mechanisms: Functional characterization using new analysis and information visualization methods. *Nucleic Acids Res.*, 29:1097–1106.
63. Rompf, A., Hungerer, C., Hoffmann, T., Lindenmeyer, M., Romling, U., Gross, U., Doss, M.O., Arai, H., Igarashi, Y., and Jahn, D., 1998, Regulation of *Pseudomonas aeruginosa* *hemF* and *hemN* by the dual action of the redox response regulators Anr and Dnr. *Mol. Microbiol.*, 29:985–997.
64. Dailey, T.A. and Dailey, H.A., 1998, Identification of an FAD superfamily containing protoporphyrinogen oxidases, monoamine oxidases, and phytoene desaturase. Expression and characterization of phytoene desaturase of *Myxococcus xanthus*. *J. Biol. Chem.*, 273:13658–13662.
65. Sasarman, A., Letowski, J., Czaika, G., Ramirez, V., Nead, M.A., Jacobs, J.M., and Morais, R., 1993, Nucleotide sequence of the *hemG* gene involved in the protoporphyrinogen oxidase activity of *Escherichia coli* K12. *Can. J. Microbiol.*, 39:1155–1161.
66. Nakayashiki, T., Nishimura, K., and Inokuchi, H., 1995, Cloning and sequencing of a previously unidentified gene that is involved in the biosynthesis of heme in *Escherichia coli*. *Gene*, 153:67–70.
67. Hansson, M. and Hederstedt, L., 1992, Cloning and characterization of the *Bacillus subtilis* *hemEHY* gene cluster, which encodes protoheme IX biosynthetic enzymes. *J. Bacteriol.*, 174:8081–8093.
68. Heurgue-Hamard, V., Champ, S., Engstrom, A., Ehrenberg, M., and Buckingham, R.H., 2002, The *hemK* gene in *Escherichia coli* encodes the N(5)-glutamine methyltransferase that modifies peptide release factors. *EMBO J.*, 21:769–778.
69. Nakahigashi, K., Kubo, N., Narita, S., Shimaoka, T., Goto, S., Oshima, T., Mori, H., Maeda, M., Wada, C., and Inokuchi, H., 2002, HemK, a class of protein methyl transferase with similarity to DNA methyl transferases, methylates polypeptide chain release factors, and hemK

- knockout induces defects in translational termination. *Proc. Natl. Acad. Sci. USA* 99:1473–1478.
70. Sasarman, A., Chartrand, P., Lavoie, M., Tardif, D., Proschek, R., and Lapointe, C., 1979, Mapping of a new *hem* gene in *Escherichia coli* K12. *J. Gen. Microbiol.*, 113:297–303.
 71. Hansson, M. and Hederstedt, L., 1994, *Bacillus subtilis* HemY is a peripheral membrane protein essential for protoheme IX synthesis which can oxidize coproporphyrinogen III and protoporphyrinogen IX. *J. Bacteriol.*, 176:5962–5970.
 72. Hansson, M., Gustafsson, M.C., Kannangara, C.G., and Hederstedt, L., 1997, Isolated *Bacillus subtilis* HemY has coproporphyrinogen III to coproporphyrin III oxidase activity. *Biochim. Biophys. Acta*, 1340:97–104.
 73. Panek, H. and O'Brian, M.R., 2002, A whole genome view of prokaryotic haem biosynthesis. *Microbiology*, 148:2273–2282.
 74. Al-Karadaghi, S., Hansson, M., Nikonov, S., Jonsson, B., and Hederstedt, L., 1997, Crystal structure of ferrochelatase: The terminal enzyme in heme biosynthesis. *Structure*, 5:1501–1510.
 75. Lecerof, D., Fodje, M., Hansson, A., Hansson, M., and Al-Karadaghi, S., 2000, Structural and mechanistic basis of porphyrin metallation by ferrochelatase. *J. Mol. Biol.*, 297:221–232.
 76. Wilks, A., 2002, Heme oxygenase: Evolution, structure, and mechanism. *Antioxid. Redox Signal.*, 4:603–614.
 77. Abraham, N.G., Drummond, G.S., Lutton, J.D., and Kappas, A., 1996, The physiological significance of heme oxygenase. *Cell. Physiol. Biochem.*, 6:129–168.
 78. Cornejo, J., Willows, R.D., and Beale, S.I., 1998, Phytobilin biosynthesis: Cloning and expression of a gene encoding soluble ferredoxin-dependent heme oxygenase from *Synechocystis* sp. PCC 6803. *Plant J.*, 15:99–107.
 79. Vasil, M.L. and Ochsner, U.A., 1999, The response of *Pseudomonas aeruginosa* to iron: genetics, biochemistry and virulence. *Mol. Microbiol.*, 34:399–413.
 80. Ochsner, U.A. and Vasil, M.L., 1996, Gene repression by the ferric uptake regulator in *Pseudomonas aeruginosa*: Cycle selection of iron-regulated genes. *Proc. Natl. Acad. Sci. USA*, 93:4409–4414.
 81. Caignan, G.A., Deshmukh, R., Wilks, A., Zeng, Y., Huang, H.W., Moenne-Loccoz, P., Bunce, R.A., Eastman, M.A., and Rivera, M., 2002, Oxidation of heme to beta- and delta-biliverdin by *Pseudomonas aeruginosa* heme oxygenase as a consequence of an unusual seating of the heme. *J. Am. Chem. Soc.*, 124:14879–14892.
 82. Bhoo, S.-H., Davis, S.J., Walker, J., Karniol, B., and Viestra, R.D., 2001, Bacteriophytochromes are photochromic histidine kinases using a biliverdin chromophore. *Nature*, 414:776–779.
 83. Wegele, R. and Frankenberg, N., unpublished results.
 84. Murphy, M.J., Siegel, L.M., Tove, S.R., and Kamin, H., 1974, Siroheme: A new prosthetic group participating in six-electron reduction reactions catalyzed by both sulfite and nitrite reductases. *Proc. Natl. Acad. Sci. USA*, 71:612–616.
 85. Crane, B.R., Siegel, L.M., and Getzoff, E.D., 1995, Sulfite reductase structure at 1.6 Å: Evolution and catalysis for reduction of inorganic anions. *Science*, 270:59–67.
 86. Crane, B.R. and Getzoff, E.D., 1996, The relationship between structure and function for the sulfite reductases. *Curr. Opin. Struct. Biol.*, 6:744–756.
 87. Warren, M.J., Bolt, E.L., Roessner, C.A., Scott, A.I., Spencer, J.B., and Woodcock, S.C., 1994, Gene dissection demonstrates that the *Escherichia coli* *cysG* gene encodes a multifunctional protein. *Biochem. J.*, 302(Pt 3):837–844.
 88. Spencer, J.B., Stolowich, N.J., Roessner, C.A., and Scott, A.I., 1993, The *Escherichia coli* *cysG* gene encodes the multifunctional protein, siroheme synthase. *FEBS Lett.*, 335:57–60.
 89. Raux, E., McVeigh, T., Peters, S.E., Leustek, T., and Warren, M.J., 1999, The role of *Saccharomyces cerevisiae* Met1p and Met8p in sirohaem and cobalamin biosynthesis. *Biochem. J.*, 338(Pt 3):701–708.

90. Schubert, H.L., Raux, E., Brindley, A.A., Leech, H.K., Wilson, K.S., Hill, C.P., and Warren, M.J., 2002, The structure of *Saccharomyces cerevisiae* Met8p, a bifunctional dehydrogenase and ferrochelatase. *EMBO J.*, 21:2068–2075.
91. Leech, H.K., Raux-Deery, E., Heathcote, P., and Warren, M.J., 2002, Production of cobalamin and sirohaem in *Bacillus megaterium*: An investigation into the role of the branchpoint chelataases sirohydrochlorin ferrochelatase (SirB) and sirohydrochlorin cobalt chelatase (CbiX). *Biochem. Soc. Trans.*, 30:610–613.
92. Roth, J.R., Lawrence, J.G., and Bobik, T.A., 1996, Cobalamin (coenzyme B₁₂): Synthesis and biological significance. *Annu. Rev. Microbiol.*, 50:137–181.
93. Banerjee, R., 1999, *Chemistry and Biochemistry of B₁₂*, John Wiley and Son, New York.
94. Debussche, L., Thibaut, D., Cameron, B., Crouzet, J., and Blanche, F., 1993, Biosynthesis of the corrin macrocycle of coenzyme B₁₂ in *Pseudomonas denitrificans*. *J. Bacteriol.*, 175:7430–7440.
95. Warren, M.J., Raux, E., Schubert, H.L., and Escalante-Semerena, J.C., 2002, The biosynthesis of adenosylcobalamin (vitamin B₁₂). *Nat. Prod. Rep.*, 19:390–412.
96. Roessner, C.A., Santander, P.J., and Scott, A.I., 2001, Multiple biosynthetic pathways for vitamin B₁₂: Variations on a central theme. *Vitam. Horm.*, 61:267–297.
97. Walker, C.J. and Willows, R.D., 1997, Mechanism and regulation of Mg-chelatase. *Biochem. J.*, 327(Pt 2):321–333.
98. Warren, M.J. and Jahn, D., unpublished data.
99. Chang, C.K. 1985, On the structure of heme d1. An isobacteriochlorin derivative as the prosthetic group of dissimilatory nitrite reductase? *J. Biol. Chem.*, 260:9520–9522.
100. Weeg-Aeressens, E., Wu, W.S., Ye, R.W., Tiedje, J.M., and Chang, C.K., 1991, Purification of cytochrome cd1 nitrite reductase from *Pseudomonas stutzeri* JM300 and reconstitution with native and synthetic heme d1. *J. Biol. Chem.*, 266:7496–7502.
101. Fulop, V., Moir, J.W., Ferguson, S.J., and Hajdu, J., 1995, The anatomy of a bifunctional enzyme: Structural basis for reduction of oxygen to water and synthesis of nitric oxide by cytochrome cd1. *Cell*, 81:369–377.
102. Kawasaki, S., Arai, H., Kodama, T., and Igarashi, Y., 1997, Gene cluster for dissimilatory nitrite reductase (nir) from *Pseudomonas aeruginosa*: Sequencing and identification of a locus for heme d1 biosynthesis. *J. Bacteriol.*, 179:235–242.
103. Nakahigashi, K., Nishimura, K., Miyamoto, K., and Inokuchi, H., 1991, Photosensitivity of a protoporphyrin-accumulating, light-sensitive mutant (*visA*) of *Escherichia coli* K-12. *Proc. Natl. Acad. Sci. USA*, 88:10520–10524.
104. Doss, M. and Philipp-Dormston, W.K., 1971, Porphyrin and heme biosynthesis from endogenous and exogenous delta-aminolevulinic acid in *Escherichia coli*, *Pseudomonas aeruginosa*, and *Achromobacter metalcaligenes*. *Hoppe Seylers Z. Physiol. Chem.*, 352:725–733.
105. Jacobs, N.J., Jacobs, J.M., and Mills, B.A., 1973, Role of oxygen in the late steps of heme synthesis in pseudomonads and *Escherichia coli*. *Enzyme*, 16:50–56.
106. Woodard, S.I. and Dailey, H.A., 1995, Regulation of heme biosynthesis in *Escherichia coli*. *Arch. Biochem. Biophys.*, 316:110–115.
107. Verderber, E., Lucast, L.J., Van Dehy, J.A., Cozart, P., Etter, J.B., and Best, E.A., 1997, Role of the *hemA* gene product and delta-aminolevulinic acid in regulation of *Escherichia coli* heme synthesis. *J. Bacteriol.*, 179:4583–4590.
108. Philipp-Dormston, W.K. and Doss, M., 1973, Comparison of porphyrin and heme biosynthesis in various heterotrophic bacteria. *Enzyme*, 16:57–64.
109. Krieger, R., Rompf, A., Schobert, M., and Jahn, D., 2002, The *Pseudomonas aeruginosa* *hemA* promoter is regulated by Anr, Dnr, NarL and Integration Host Factor. *Mol. Genet. Genomics*, 267:409–417.

110. Arai, H., Kodama, T., and Igarashi, Y., 1997, Cascade regulation of the two CRP/FNR-related transcriptional regulators (ANR and DNR) and the denitrification enzymes in *Pseudomonas aeruginosa*. *Mol. Microbiol.*, 25:1141–1148.
111. Hasegawa, N., Arai, H., and Igarashi, Y., 1998, Activation of a consensus FNR-dependent promoter by DNR of *Pseudomonas aeruginosa* in response to nitrite. *FEMS Microbiol. Lett.*, 166:213–217.
112. Wang, L.Y., Brown, L., Elliott, M., and Elliott, T., 1997, Regulation of heme biosynthesis in *Salmonella typhimurium*: Activity of glutamyl-tRNA reductase (HemA) is greatly elevated during heme limitation by a mechanism which increases abundance of the protein. *J. Bacteriol.*, 179:2907–2914.
113. Wang, L., Elliott, M., and Elliott, T., 1999, Conditional stability of the HemA protein (glutamyl-tRNA reductase) regulates heme biosynthesis in *Salmonella typhimurium*. *J. Bacteriol.*, 181:1211–1219.
114. Wang, L., Wilson, S., and Elliott, T., 1999, A mutant HemA protein with positive charge close to the N terminus is stabilized against heme-regulated proteolysis in *Salmonella typhimurium*. *J. Bacteriol.*, 181:6033–6041.
115. Jahn, D., Michelsen, U., and Söll, D., 1991, Two glutamyl-tRNA reductase activities in *Escherichia coli*. *J. Biol. Chem.*, 266:2542–2548.
116. Javor, G.T. and Febre, E.F., 1992, Enzymatic basis of thiol-stimulated secretion of porphyrins by *Escherichia coli*. *J. Bacteriol.*, 174:1072–1075.

PRODUCTION OF CYCLIC LIPOPEPTIDES BY FLUORESCENT PSEUDOMONADS

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1. INTRODUCTION

Members of the genus *Pseudomonas* produce a large amount of metabolites that are released into the extracellular environment. Among these metabolites are siderophores, phytohormones, biosurfactants and several antibiotic compounds, which may have broad-spectrum activity^{24, 81}. Production of cyclic lipopeptides (CLPs) including compounds referred to as lipodepsipeptides, lipoundecapeptides and so on, has recently been established as a common trait among the pseudomonads. However, CLPs are also produced by a variety of other bacteria as well as fungi. Among the bacterial metabolites the CLP surfactin produced by *Bacillus subtilis* has received large attention due to its powerful biosurfactant activity and the antifungal, antibacterial and antiviral effects⁷⁸.

The CLPs constitute a large group of structurally related compounds. The common feature is a fatty acid coupled to the N-terminal of a relatively short oligopeptide, which is cyclized by the formation of a lactone ring between two of the amino acids. The compounds within this group are however extremely variable due to differences in the fatty acid (length and

modifications), type (note that many unusual amino acids occur in this type of molecules), number, and modification of amino acids and organization of the lactone ring. Please refer to Figure 1 for an illustration of CLP structure.

This chapter will deal with the CLPs produced by different strains of the genus *Pseudomonas*, and focus on their structural and functional properties. Some CLPs produced by *Pseudomonas syringae* or *Pseudomonas fuscovaginae* are particularly well studied as phytotoxins. A detailed review of the phytotoxins will be provided in a separate chapter. We will include information on phytotoxins if relevant information is not available for other *Pseudomonas* compounds, and whenever needed to compare the properties of different *Pseudomonas* CLPs.

2. THE STRUCTURE OF CLPs PRODUCED BY *PSEUDOMONAS*

Pseudomonads are able to produce a diverse array of CLPs. Nevertheless, it appears that the ones identified so far can be assigned to four major groups, each comprising compounds displaying significant structural similarities but also some heterogeneity.

2.1. The Viscosin Group

The viscosin group presently contains the following CLPs: viscosin⁵⁷, the white line inducing principle (WLIP)⁶⁷, the massetolids³¹, viscosinamide⁷², the pseudophomins⁷⁹ and a related, but not fully characterized CLP produced by *Pseudomonas extremorientalis*⁵¹ (Table 1).

Production of viscosin-group CLPs seems to be a property associated with several fluorescent pseudomonads isolated from different environments including drinking water, surfaces of marine algae, surfaces of mushrooms, decayed broccoli and sugar beet rhizosphere. The pseudophomins have been isolated from a *Pseudomonas fluorescens* strain⁷⁷ while viscosin production has been reported from strains of *P. fluorescens* biovar II and possibly biovar IV^{45, 57}. However, more extensive surveys have revealed that viscosinamide and closely related compounds are produced by biovar I, and biovar V strains from rhizosphere^{71, 72, 74}. This might indicate that CLP production occurs in defined taxonomic groups within the *Pseudomonas* complex. Comparable results have previously been obtained for production of the antibiotic DAPG⁵², which is produced by strains belonging to biovars II and IV according to Nielsen *et al.*⁷¹.

WLIP is a CLP of *Pseudomonas "reactans,"* an ill-defined species closely related to *Pseudomonas tolaasii*⁹¹. Interestingly, the nearest phylogenetic

Table 1. Simplified primary structures of CLPs produced by *Pseudomonas* sp.

<i>Viscosin group</i>	
Viscosin	3-HDA-L-Leu-D-Glu-D- <i>allo</i> Thr-D-Val-L-L-Leu-D-Ser-L-Leu-D-Ser-L-Ile
Viscosin-amide	3-HDA-L-Leu-D-Gln-D- <i>allo</i> Thr-D-Val-L-L-Leu-D-Ser-L-Leu-D-Ser-L-Ile
Massetolide D	3-HDA-L-Leu-D-Glu-D- <i>allo</i> Thr-D-Ile-L-L-Leu-D-Ser-L-Leu-D-Ser-L-Leu
WLIP	3-HDA-L-Leu-D-Glu-D- <i>allo</i> Thr-D-Val-D-L-Leu-D-Ser-L-Leu-D-Ser-L-Ile
Pseudophomin A	3-HDA-L-Leu-D-Glu-D- <i>allo</i> Thr-D-Ile-D-L-Leu-D-Ser-L-Leu-D-Ser-L-Ile
<i>Amphisin group</i>	
Amphisin	3-HDA-D-Leu-D-Asp-D- <i>allo</i> Thr-D-Leu-D-Leu-D-Ser-L-Leu-D-Gln-L-Leu-L-Ile-L-Asp
Tensin	3-HDA-D-Leu-D-Asp-D- <i>allo</i> Thr-D-Leu-D-Leu-D-Ser-L-Leu-D-Gln-L-Leu-L-Ile-L-Glu
Pholipeptin A	3-HDA-D-Leu-L-Asp- <i>----</i> L-Thr-D-Leu-D-Leu-D-Ser-D-Leu-L-Ile-D-Asp
Lokisin	3-HDA- \otimes -Leu- \otimes -Asp-D- <i>allo</i> Thr- \otimes -L-Leu- \otimes -Ser- \otimes -Leu-L-Ile- \otimes -Asp
<i>Tolaasin group</i>	
Tolaasin	3-HOA-Dhb-Pro-Ser-Leu-Val-Ser-Leu-Val-Val-Gln-Leu-Val-Dhb- <i>allo</i> Thr-Ile-Hse-Dab-Lys
FP-B	3-HOA-Dhb-Pro-Leu-Ala-Ala-Ala-Val-Gly-Ala-Val-Ala-Val-Dhb- <i>allo</i> Thr-Ala-Dab-Dab-Phe
SP-22A	3-HDA-Dhb-Pro-Val-Val-Ala-Ala-Val-Val-Dhb-Ala-Val-Ala-Ala-Dhb- <i>allo</i> Thr-Ser-Ala-Dhb-Dab-Tyr
CP-A	3-HDA-Dhb-Pro-Ala-Ala-Ala-Val-Val-Dhb-Hse-Val- <i>allo</i> lle-Dhb-Ala-Ala-Val-Dhb- <i>allo</i> Thr-Ala-Dab-Ser-Ile
<i>Syringomycin group</i>	
Pseudomycin A	3,4-DTDA-L-Ser-D-Dab-L-Asp-L-Lys-L-Dab-L- <i>allo</i> -Thr-Z-Dhb-L-Asp(3-OH)-L-Thr(4-Cl)
Syringomycin E	3-HDA- <i>---</i> L-Ser-D-Ser-D-Dab-L-Dab-L-Arg- <i>----</i> L-Phe-Z-Dhb-L-Asp(3-OH)-L-Thr(4-Cl)
Syringostatins	3-HXX- <i>---</i> L-Ser-D-Dab-L-Dab-D-Hse-L-Orn-L- <i>allo</i> -Thr-Z-Dhb-L-Asp(3-OH)-L-Thr(4-Cl)
Syringotoxin	3-HTA- <i>---</i> L-Ser-D-Dab-L-Asp-L-Lys-L-Dab-L- <i>allo</i> -Thr-Z-Dhb-L-Asp(3-OH)-L-Thr(4-Cl)

Hydrophobic amino acids are shown in blue, non-ionizable polar in green, ionizable basic in red and ionizable acidic in black. \otimes : isomer not identified; Dab: 2,4-diaminobutyric acid; Dnb: 2,3-dihydro-2-aminobutyric acid; Hse: homoserine; FP-B: fuscopепtin B; SP-22A: syringopeptin 22A; CP-A: corpeptin A; 3-HDA: 3-hydroxy-decanoic acid; 3-HOA: 3-hydroxy-octanoic acid; 2,3-DTDA: 2,3-dihydroxy-tetradecanoic acid; 3-HTA: 3-hydroxy-tetradecanoic acid, 3-HXX: either 3-hydroxy dodecanoic acid or 3-hydroxy-tetradecanoic acid.

relatives of *P. extremorientalis* include *P. tolaasii*⁵¹. Possibly production of viscosin-group compounds is even more widespread, since the massetolids were isolated from a marine pseudomonad that could not be identified to the species level³¹. Furthermore Ivanova and co-workers⁵¹ reported the production of “low-molecular-mass peptides with surface-active properties” by *Pseudomonas veronii*, *Pseudomonas rhodesiae* and *Pseudomonas orientalis*. On-going work to characterize the genes involved in CLP production (see Section 3 below) has led to the development of degenerated PCR-primers targeting peptide synthetase loci involved in CLP production in *P. fluorescens*⁸⁵. Possibly these primers might even prove useful to identify novel CLP producers by applying molecular screening strategies.

All the above CLPs are composed of nine amino acids, including several D-amino acids and they share a number of structural features. Every one contains a majority of hydrophobic amino acids at positions 1, 4, 5, 7 and 9, supplemented by uncharged polar amino acids at positions 3, 6 and 8 and an ionizable acidic amino acid at position 2. Viscosinamide is an exception to this general rule as the acidic amino acid D-Glu is substituted by the uncharged D-Gln. The sequence of hydrophobic and non-ionizable polar amino acids in the peptide moiety represents a conserved motif as indicated in Table 1. The peptide is made circular by a lactone formed by an ester bond between the –CO group of the C-terminal amino acid and the D-*allo*-Thr-OH group so that the cyclized part of the molecules consists of seven amino acids^{31, 57, 67, 72, 79}. The peptides are linked at the N-terminus to a lipid, which in most cases is a 3-hydroxy decanoyl, although for pseudophomin B a 3-hydroxy dodecanoic acid constitutes the lipid residue⁷⁹.

The structure of WLIP has been determined by x-ray crystallographic methods, which revealed that the peptide part of the molecule is folded in a so-called β -turn type form II⁴¹. According to subsequent analysis of these data by Sørensen⁹⁷ the amphiphilic properties of the molecule is revealed from the collection of hydrophobic amino acids in one region of the molecule and hydrophilic or acidic amino acids in two other regions. The conformation of WLIP in DMSO- d_6 has been investigated by nuclear magnetic resonance (NMR) spectroscopy and by molecular modelling, which revealed that the overall fold of the lactone ring resembles the seam of a tennis ball⁶⁷. Recently, the chemical structures of the pseudophomins have been determined to be similar to that of WLIP^{77, 79}.

The CLPs belonging to the viscosin group are generally referred to as water-soluble, extracellular compounds. However, viscosinamide has a solubility in water about $2 \mu\text{mol l}^{-1}$, which is about 5 times lower than that of viscosin, and it appears to be tightly bound to the cell wall or membrane⁷². As mentioned above the only difference between viscosin and viscosinamide is a Glu/Gln exchange at amino acid position 2. A similar Glu/Gln exchange at

amino acid position 1 is found for the *Bacillus* CLPs surfactin and lichenysin, where it also results in an increased association of the Gln-containing compound with biological membranes³³.

2.2. The Amphisin Group

At present the CLPs amphisin, tensin, lokisin and pholipeptin are well-characterized members of this group^{44, 73, 96, 98, 102}, which even includes the less well-characterized compound hodersin⁷⁴. Amphisin, tensin and lokisin are produced by members of *P. fluorescens* biovar VI, according to the survey performed by Nielsen *et al.*⁷⁴. Isolates producing amphisin and lokisin were relatively rare while tensin production was documented for 29% of the rhizosphere *Pseudomonas* strains included in the survey.

The amphisin-group CLPs resemble members of the viscosin group, but contain 11 amino acids coupled to a 3-hydroxy decanoic fatty acid. Although several amino acid substitutions has occurred between the members of the amphisin group, the sequence motif of hydrophilic and hydrophobic amino acids resembles that found for viscosin-like molecules (Table 1). The lactone is formed by the C-terminal amino acid and the D-*allo*-Thr or L-Thr residue found at amino acid position 3^{44, 96, 98, 102}. The structure of amphisin is shown in Figure 1A.

The chemical, including crystal, structures of tensin and amphisin have been solved. The secondary structure explains the amphiphilic properties of these molecules: hydrophobic and hydrophilic amino acids are arranged to form a hydrophobic “bottom side” and a hydrophilic “upper side” from where the 3-hydroxydecanoic acid protrudes as illustrated for amphisin in Figure 1B⁹⁷. The peptide backbones of the molecules are essentially helical and contain a 3₁₀ helix from Leu at amino acid position 2 to Leu at position 8. For lokisin less information is available, but GC and NMR analyses have confirmed that the molecule is different from pholipeptin^{98, 102}.

2.3. The Tolaasin Group

This group of CLPs consists of relatively large molecules containing between 19 and 25 amino acids. The representatives of this group are the corpeptins, the fuscopeptins, the syringopeptins and the tolaasins (Table 1)^{6, 26, 66, 92}. The corpeptins, the fuscopeptins and the syringopeptins are recognized phyto-toxins, while tolaasin has been found to cause disease on the mushroom *Agaricus bisporus* (Section 5).

P. tolaasii, which may be endemic to compost is currently the best characterized producer of tolaasin⁹². Recently, a PCR-assay that targeted *P. tolaasii* genes involved in tolaasin synthesis permitted specific detection of this

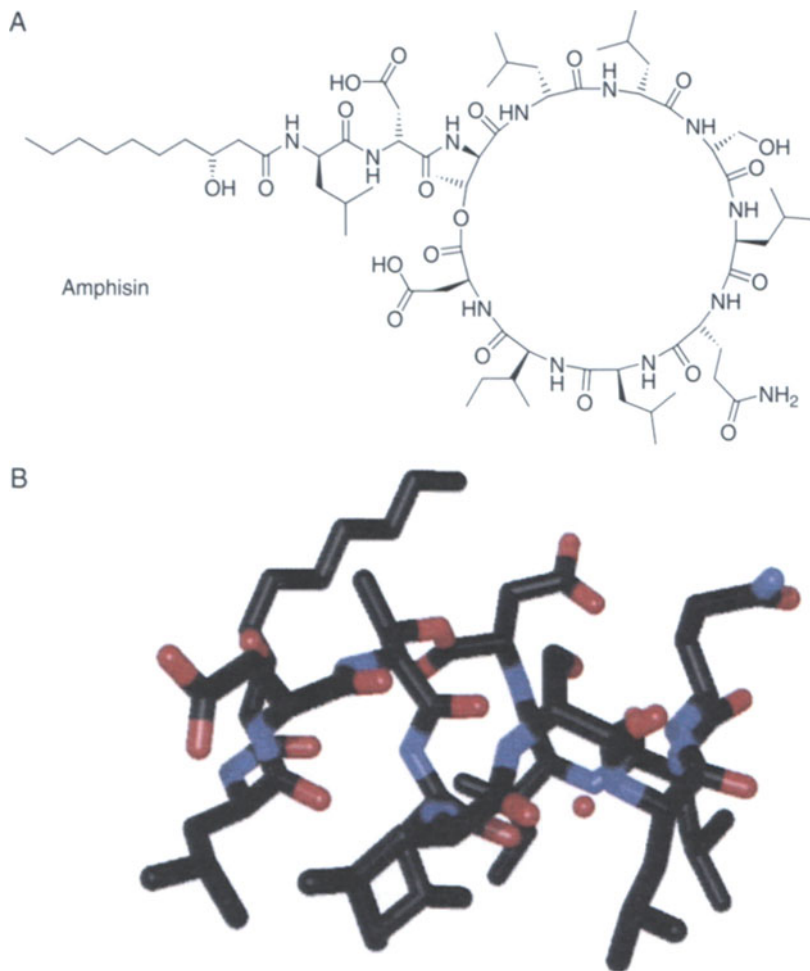


Figure 1. (A) The primary structure of amphisin. (B) The secondary structure of amphisin. The fatty acid tail is seen in the upper left (black). Hydrophobic amino acids are at the bottom of the structure (black). Amino acids with hydrophilic groups (red) protrude at the top. The dot represents a water molecule. The figures are obtained from ref. [97] with kind permission from Dr. Dan Sørensen.

species⁵⁸. However, using the white-line-in-agar test, which is based on an interaction between WLIP and tolaasin, Munsch and Alatossava⁶⁹ as well as Godfrey *et al.*³² suggested that other organisms, for example, *Pseudomonas costantinii* and a species genetically related to *P. syringae* also produce tolaasin. It is possible that the above microorganisms do not produce tolaasin but closely related compounds, since the white-line-in-agar assay may not be specific enough⁶⁹.

Corpeptins, fuscopeptins and syringopeptins are produced by plant pathogenic strains of *Pseudomonas corrugata*, *P. fuscovaginae* and *P. syringae*, respectively^{6, 26, 36}. Screening for novel strains producing these CLPs are likely to be facilitated by the development of PCR-assays as mentioned above for tolaasin, as well as by immunological assays as shown recently for syringopeptins²⁸.

CLPs in the tolaasin group are all found in many variants due to amino acid replacements or differences in the lipid moiety. Most often the acyl moiety is 3-hydroxydecanoyl, but in tolaasin a 3-hydroxyoctanoic acid is linked to the N-terminus of the peptide. Corpeptin B is unique for the fact of being acylated by an unsaturated fatty acid, *cis*-3-hydroxy-5-dodecenoyl. The peptide moieties of these CLPs are hydrophobic with a positively charged C-terminus⁸. The peptide part contains several D-forms and a number of “unusual” amino acids as homoserine (Hse), 2,4-diamino butyric acid (Dab) and 2,3-dihydro-2-aminobutyric acid (Dhb). For tolaasin the sequence motif involving alternating hydrophobic and non-ionizable polar amino acids, which was described for the viscosin and amphisin groups above, appears in the N-terminal part of the peptide (Table 1). The peptide is cyclized by a lactone formed between the C-terminal and the –OH group of *allo*-Thr residue as seen for the members of the viscosin and amphisin groups. Consequently the cyclic parts of the molecules contain 5–8 amino acids. Another conserved trait is that *allo*-Thr is preceded by Dhb in all members of this CLP group.

Structural analyses have been carried out for several of these CLPs employing circular dichroism (CD) and NMR spectroscopy, mass spectrometry etc.^{5, 6, 8, 26, 36, 66}. According to Baré *et al.*⁸ the above studies indicate that these CLPs, except syringopeptin 25A, are mainly unstructured or partly structured in aqueous environments. However in solutions containing SDS or trifluoroethanol to generate a membrane-like environment several structural β -turn and γ -turn motifs occur in fuscopeptins as well as in syringopeptins. Apparently, relatively rigid Dhb amino acid residues delineate different structural regions of these molecules^{5, 8}. For example, a region of the tolaasin, fuscopeptin and syringopeptin molecules forms an (left handed) α -helix while the overall conformation of the lactone ring resembles the “seam of a tennis ball structure” that has been reported for WLIP, a member of the viscosin group^{5, 8, 66}.

The ability of several CLPs to form structures in membrane-like environments, but not in water is in agreement with the ability of CLPs to insert into and perturb membranes as noted by Baré *et al.*⁸. Analyses of the conformational properties of CLPs are made difficult by the fact that these molecules can adopt a number of distinct and different conformations, in part depending on the solvent⁴⁴. However, it appears likely that this conformational flexibility might be important for the interactions between CLPs and biological membranes⁹⁷.

2.4. The Syringomycin Group

This last group of *Pseudomonas* CLPs contains several well-characterized phytotoxins: the syringomycins, the syringostatins and the syringotoxins^{10, 30, 90} (see *Pseudomonas* Volume 2, Chapter 4). Furthermore the pseudomycins and the ecomycins, originally isolated as antifungal compounds, are members of this group^{3, 61}. The syringomycins, syringostatins and pseudomycins have all been isolated from the plant pathogen *P. syringae*^{10, 22} whereas syringotoxins have been found from both *P. syringae* and *P. fuscovaginae*^{3, 27, 48}. The ecomycins originate from *Pseudomonas viridiflava* known as a plant-associated saprophyte or a weak plant pathogen⁶¹. As for some of the above CLP groups, PCR primers for detection of *P. syringae* strains producing phytotoxic CLPs have been developed^{16, 95} and complement screening based on chemical analysis⁶⁴.

All the above CLPs contain nine amino acids as the viscosin-like compounds, yet the amino acid composition is very different. The “unusual” amino acids Hse, Dab and Dhb are common as in the larger hydrophobic CLPs mentioned in the previous section, but additionally L-Asp(3-OH), L-Thr(4-Cl) and L-Ornithine are found in all or some of these compounds. With the possible exception of the ecomycins, the amino acids in positions 1, 7, 8 and 9 are conserved among the compounds¹⁰. The lactone ring is formed between the N-terminal Ser and the C-terminal Thr(4-Cl), which is quite different from the ring closure seen in other *Pseudomonas* CLPs. In spite of these differences, the conformation of the ring includes rigid structural regions interspaced by more flexible regions yielding a “seam of tennis ball” structure as seen for other pseudomonad CLPs^{8, 18}.

The syringomycin group CLPs are linked at the N-terminal amino acid to a 3-hydroxy or 3,4-dihydroxy fatty acid that may contain 10, 12 or 14 carbon atoms^{4, 5, 61, 90}. Hence differences in the lipid moiety contribute significantly to the heterogeneity of this group of CLPs.

3. THE MOLECULAR BACKGROUND FOR CLP PRODUCTION

Pseudomonas CLPs are produced on large, multi-functional enzymes named peptide synthetases. These enzymes employ the so-called multicomponent template mechanism for non-ribosomal peptide synthesis, which is generally recognized as less specific than ribosomal protein synthesis²³. The low specificity can explain why pseudomonads may produce less abundant CLP compounds in addition to their major product⁷⁴ and why CLP producing strains generate novel CLP analogues when fed with specific, non-protein amino acids³¹.

The peptide synthetases are organized in domains collected in modules that can catalyse the reactions necessary for the formation of an elongating peptide product^{23, 54}. In brief, specific enzymatic domains recognize the amino acid substrates and activate them by adenylation. These unstable intermediates, which are associated with the enzyme, are subsequently stabilized by thioesterification carried out by a thiolation domain. Finally, peptide bonds are formed between activated precursors by a condensation domain. In addition to these domains, specific parts of the enzyme may be involved in, for example, epimerization of α -carbon atoms, cyclization reactions and the final release of the peptide product from the peptide synthetase. Several of the domains carry conserved amino acids motifs, for example, the adenylation and thiolation domains, which facilitate identification of these enzymes from incomplete sequence data (e.g., Koch *et al.*⁵³).

The chromosomal region involved in synthesis of syringomycin covers about 55 kb and is presently the best characterized *Pseudomonas* peptide synthetase gene cluster. The cluster contains at least six genes, *syrD*, *syrP*, *syrB1*, *syrB2*, *syrC* and *syrE*. The *syrB1* and *syrE* genes encode peptide synthetase modules and *syrC* codes for a thioesterase-like enzyme. *Syr D* is a putative ABC transporter protein while the *syrP* and possibly the *salA* gene products have regulatory functions^{39, 88, 104}. Interestingly, a *syrB*-like gene seems to be involved in production of syringomycin, syringostatin as well as syringotoxin, as primers amplifying this region have been used to identify *P. syringae* strains producing all of these CLPs⁹⁵.

Recently a chromosomal region of at least 73 kb has been identified, which is involved in synthesis of syringopeptin 22 (see ref. [89]). Within this region, three genes, *sypA*, *sypB* and *sypC* encode proteins with homology to peptide synthetases, while a fourth gene encodes an ABC transporter that may be involved in export of syringomycin as well as of syringopeptin^{87, 88}. Even for another of the large, hydrophobic CLPs, tolaasin, three high molecular weight proteins, which probably are peptide synthetases, have been found to be required for CLP production⁸⁴. At least one tolaasin synthetase is distinct from other *Pseudomonas* synthetases. Hence, Lee *et al.*⁵⁸ were able to design primers targeting a biosynthetic gene that only amplified DNA from *P. tolaasii* but not from several other fluorescent pseudomonads, including *P. syringae*. Furthermore, Godfrey *et al.*³² compared a 188 bp sequence of "tolaasin synthetase" in a *P. syringae*-like tolaasin producer with *P. tolaasii* and *P. syringae* sequences, and found similarities of about 72% between "tolaasin synthetase" and *syrE*.

In a viscosin-producing *P. fluorescens* strain, a 25-kb chromosomal region involved in CLP production has been identified, and again three high molecular weight proteins that possibly constitute a peptide synthetase complex have been found to be essential¹¹. Less is known about genes involved in

synthesis of amphisin-group CLPs, but recently a protein with 54% similarity to *syrE* over a stretch of 590 amino acids was identified as an amphisin synthetase⁵³.

4. REGULATION OF CLP PRODUCTION

The production of CLPs in *Pseudomonas* are generally affected by growth phase, carbon source, nutrients and specific signal molecules; yet the specific impact that these factors have on production differ considerably among *Pseudomonas* strains producing different CLP compounds.

Viscosinamide is produced during growth of *P. fluorescens* and no production has been found in stationary phase cultures or in cultures starved for carbon or nitrogen or phosphorus⁷². Production of amphisin and tensin likewise occur in the exponential growth phase^{53, 73}. Increasing the concentrations of C, N or P sources in growth media leads to an improved production of viscosinamide and tensin, but only as long as the rising substrate or nutrient levels support increased growth^{72, 73}. Hence, as production of these CLPs is tightly coupled to cell proliferation, they cannot be considered as secondary metabolites. In contrast syringomycin and syringopeptin are produced in the stationary phase^{35, 37}, while data for tolaasin production are ambiguous⁹².

Some investigations have addressed how the composition of growth media affects CLP production, but the data are too scarce to allow clear comparisons. The significance of the carbon source has been illustrated for a tensin-producing *P. fluorescens* strain⁷³, as well as for phytotoxin production by *P. syringae*¹⁰³. A comparable study focussing on N-sources, demonstrated a high syringomycin production by *P. syringae* grown on histidine³⁷. The significance of phosphate for CLP production is variable as levels above 1 mM inhibit syringomycin production but has no negative effect on tensin production^{37, 73}. Finally it has been observed that more than 2 μ M of iron is required for syringomycin production³⁷.

Syringomycin production as well as the expression of *syrB* is stimulated by plant signal molecules including phenolics and flavenoid glycosides^{62, 63, 80, 104}. The responses to signal molecules are enhanced by some sugars including D-fructose, D-mannose and sucrose^{62, 63}. Interestingly, plant-derived signal molecules may play a role in amphisin production, but the active compound(s) is different from those that stimulate syringomycin production⁵³.

The molecular mechanisms involved in regulation of CLP production are far from being fully understood, and most of the information available relative to genes involved in phytotoxin production is on *P. syringae*. Involvement of the Gac two-component system⁴³ seems to be a common regulatory theme documented for amphisin, tolaasin and syringomycin production^{34, 47, 53}.

Spontaneous mutations occurring in the *gacS* or *gacA* genes lead to instable expression of CLPs and other Gac-regulated traits in *P. tolaasii* as well as in other pseudomonads^{15, 40}.

The plant signal affecting amphisin production appear to be channelled through the Gac regulatory system, whereas the plant signals that stimulate syringomycin synthesis do not seem to activate this global regulatory system^{62, 63}. Quorum sensing mediated by *N*-acylhomoserine lactones that regulates the expression of several exo-products in plant-associated pseudomonads controls neither syringomycin nor amphisin production^{2, 10}.

5. FUNCTIONS OF *PSEUDOMONAS* CLPs

Pseudomonas CLPs constitute, as specified above, a complex group of compounds that have various biological functions such as biosurfactant activity, but also as antibiotic compounds with antibacterial, antifungal and/or phytotoxic actions. In this section we will initially address the significance of biosurfactants for the ability of the producing bacteria to condition their environment. Subsequently we will deal with the antimicrobial effect of the CLPs.

5.1. CLPs as Biosurfactants and Chelating Agents

The four groups of amphiphilic *Pseudomonas* CLPs include powerful biosurfactants. For example, a saturated solution of viscosin lowers the surface tension of water from 71 to 25 mN/m⁵⁷. For viscosinamide and related compounds a comparable reduction of the surface tension of defined growth media from 71 to 25–27 mN/m has been reported⁷⁴. Viscosin exerts its action at low concentrations close to the critical micelle concentration (CMC) of 4 µg/ml or about 4 µM—the CMC for viscosinamide and other related compounds have not been determined. Hence, viscosin and viscosinamide appear to be as powerful biosurfactants as the well-known surfactin produced by *B. subtilis*, which lowers the surface tension of water to 27 mN/m at concentrations down to 20 µM⁷⁸. Interestingly, viscosin can accumulate in growth media at concentrations exceeding its solubility in water, possibly due to binding to acidic polysaccharides released by the producing strain⁵⁷. A comparable ability to bind to carbohydrates as β-1,3-glycans and chitin has been noted for syringomycin²¹.

CLPs from the amphisin group are slightly less potent biosurfactants than the viscosin-group compounds yielding values of 27 mN/m in comparable assays. For comparison, a mutant strain deficient in amphisin production decreased the surface tension to about 48 mN/m⁷⁴. Finally, the phytotoxins syringomycin and syringopeptin, which have CMC values around 1 mg/ml display biosurfactant properties as shown by a lowering of the interfacial

tension of water to 31 and *c.* 35 mN/m, respectively at concentrations above 10 $\mu\text{g/ml}$ ^{49, 50}.

The relation between the structures of *Pseudomonas* CLPs and their functions as biosurfactants has not been analysed in depth. However such tests for the *Bacillus* CLPs have revealed that an intact lactone ring is an essential structural trait⁶⁵.

The CLPs produced by *Bacillus* species are able to chelate cations, for example, Ca^{2+} and Mg^{2+} (see refs [33], [86]). Comparison of the properties of surfactin and lichenysin, differing by a Glu/Gln exchange at amino acid position 1, has shown that lichenysin has a higher surfactant power and is a better chelating agent than surfactin. This highlights that the polarity of amino acids in the peptide part of the surfactant molecules is highly significant for their functions³³. It has not been investigated whether *Pseudomonas* CLPs chelate cations. A comparison of the properties of viscosin and viscosinamide, which differ by a Glu/Gln exchange as mentioned above, would be particularly interesting.

5.2. Influences of CLPs on Surface Motility

Biosurfactants play an important role for bacterial surface mobility *in vitro*. In accordance, ref. [74] found a strong correlation between CLP production and surface mobility among fluorescent pseudomonads isolated from rhizosphere. This survey revealed that strains producing CLPs of the amphisin group had higher surface mobility on the tested media than strains producing viscosin-like compounds. For a selected strain, the amphisin producer *Pseudomonas* sp. DSS73, studies involving an ampicillin-deficient transposon mutant demonstrated that production of as little as 2.5 $\mu\text{g/ml}$ of this CLP is required for surface mobility on soft agar². However several CLPs from the amphisin groups (tensin) as well as from the viscosin group (viscosinamide) and serrawettin W (produced by *Serratia*) could restore surface mobility in the mutant strain. This shows that both cell-associated CLPs as viscosinamide and soluble compounds as amphisin can be involved in surface mobility. Unfortunately, the significance of these compounds for motility on well-defined hydrophobic surfaces has not been tested *in vitro*. An important result from the work by Andersen and co-workers was that *in vitro* containment of the root-pathogenic fungi *Rhizoctonia solani* and *Pythium ultimum* by the antagonistic strain DSS73 required surface motility. Hence, elimination of the fungi in this artificial system was due to the synergistic effects of surface motility and synthesis of several antifungal compounds.

5.3. *Pseudomonas* CLPs as Antibiotics

The *Pseudomonas* CLPs possess antagonistic actions against several prokaryotic and eukaryotic organisms and it is recognized that plasma

membranes constitute the primary target of these compounds. However, as recently pointed out by Fogliano and co-workers²⁹ the access of CLPs to the plasma membrane may be hindered by structures as cell walls in Gram-positive bacteria, fungi and plants and by outer membranes in Gram-negative bacteria. The biological function of the CLPs is therefore governed by their ability to reach the target membrane as well as their specific interactions with the membrane. Following sections will address how CLPs interfere with naked biological or artificial membranes and deal with CLP effects on intact microorganisms in vitro.

5.3.1. *Interference with Biological or Model Lipid Membranes*

The mechanism responsible for the ability of CLPs to perturb membranes has been particularly well investigated for members of the tolaasin and syringomycin groups. The syringopeptins and tolaasins as well as syringomycins and syringotoxins form channels in different types of artificial lipid membranes^{1, 12, 19, 20, 50}. The channels have a radius of about 1 nm and are formed by CLP oligomers. It has been observed that syringopeptins increase the passive permeability of artificial liposomes to better than syringomycin E¹⁷. Correspondingly the channel-forming ability of syringopeptin 22A, which forms small oligomers, is about 10 times higher than that of syringomycins E, which forms larger oligomers¹. Formation of pores is apparently the mechanism behind the ability of the above CLPs to function as cellular toxins at very low concentrations, for example, 0.2 μM for tolaasin and 0.5 μM for syringomycin E. The pores increase the influx of H^+ and Ca^{2+} as well as the efflux of K^+ leading to a collapse of the pH gradient across the membrane and induction of Ca^{2+} -mediated signalling pathways¹⁰. Possibly, the perturbation of membrane structure or the induction of signalling cascades inside the cell is the background for the impact of the CLP toxins on plasma membrane H^+ -ATPase^{9, 22} as well as the effect of pholipeptin (amphisin-group CLP) on phosphatidylinositol-specific phospholipase C¹⁰².

When tested on “naked” membranes as found, for example, on red blood cells, the haemolytic ability of tolaasin, syringopeptin, syringomycin, syringostatin and syringotoxin ranged from concentrations of 0.6 μM (syringopeptin 22A) to 6 μM (syringomycins and syringotoxins)^{1, 12, 19, 20, 49, 94}. Viscosin lyses erythrocytes at about 10 μM , which is above the CMC for this compound of about 4 μM . Therefore it is not considered to be a pore-forming toxin but to function as a non-specific detergent⁴⁶. The interactions of other viscosin or amphisin-group CLPs with membranes have not been studied in detail so far.

Viscosin inhibits or inactivates enveloped viruses as infectious bronchitis virus, influenza virus and Newcastle disease virus in vitro. Relatively high MIC values of 62–125 $\mu\text{g/ml}$ (about 50–100 μM) were reported suggesting a non-specific detergent effect³⁸. Furthermore viscosin protected embryonated

eggs against subsequent injections with infectious bronchitis virus but was not considered to have a potential as an antiviral drug in practise³⁸.

5.3.2. *In Vitro Effects on Bacteria*

Only few *Pseudomonas* CLPs have been tested systematically for effects on other bacteria. A general observation that is valid for members of all four CLP groups (viscosin, amphisin, syringopeptins and tolaasin, as well as syringomycins and syringotoxins) is that these compounds are not able to inhibit growth of intact Gram-negative bacteria^{29, 31, 56} (T.H. Nielsen and J. Sørensen, unpublished observations). However the Gram-negative plasma membrane is a target for CLP action as tolaasin disrupts protoplasts of Gram-negative organisms⁸². Accordingly, syringomycins E and syringopeptin 25A only affect growth of *P. syringae* after treatment with lysozyme²⁹. In conclusion, the Gram-negative outer membrane or peptidoglycan layer probably hinders CLP access to the plasma membrane.

Gram-positive bacteria represent an important target group for the large hydrophobic *Pseudomonas* CLPs as tolaasin, which inhibits growth of several Gram-positives⁹². Syringopeptin 22A, syringopeptin 25A and corpeptin have all been tested against *Bacillus megaterium* and inhibit growth at MIC values of 1–4 μM , while syringopeptin PhvA is considerably less potent^{26, 36, 56}. In contrast syringomycins E and syringotoxins do not inhibit growth of *B. megaterium*⁵⁶. The observation that syringopeptins are more potent antibiotics than syringomycins and syringotoxins is even valid for *Rhodococcus* and *Micrococcus* species⁵⁶. For the compounds of the viscosin and amphisin groups little information is available. However, viscosin has been reported to have no effect on *Staphylococcus aureus*³¹, while amphisin and related CLPs are active against several *Bacillus* species (T.H. Nielsen and J. Sørensen, unpublished observations).

Mycobacteria is a group within the Gram-positives that appear to be especially sensitive to *Pseudomonas* CLPs. Viscosin inhibits *Mycobacterium tuberculosis* and *Mycobacterium avium-intracellulare* at MIC values of 10–20 $\mu\text{g/ml}$ (c. 10–20 μM) and 5–10 $\mu\text{g/ml}$, respectively, while the related compound massetolid A is a slightly more potent antibiotic when tested against these species^{25, 31}. For comparison, syringopeptin 22A inhibits *Mycobacterium smegmatis* at 2–3 $\mu\text{g/ml}$ (about 1 μM) and syringomycins E is an equally potent antimycobacterial agent with a MIC value of 1.5 $\mu\text{g/ml}$ (just above 1 μM)¹³. The mycobacteria have a hydrophobic, waxy cell surface due to the presence of mycolic acid and, for *M. tuberculosis*, of glycolipids in their cell wall. Possibly this hydrophobic cell wall facilitates the passage of hydrophobic CLPs to the plasma membrane.

5.3.3. *In Vitro Effects on Fungi*

The effects that *Pseudomonas* CLPs exert on fungi have been investigated in detail for members of all four major CLP groups. Viscosinamide displays in vitro antagonistic activity against the phyto-pathogenic oomycete *Pythium ultimum* as well as the basidiomycete *Rhizoctonia solani*⁷⁴. *R. solani* hyphal growth on agar plates was reduced near filters containing viscosinamide, and aerial mycelium was not developed close to the viscosinamide source⁷². A more detailed microscopic analysis, employing a panel of fluorescent stains and activity dyes revealed that hyphae exposed to the CLP have increased branching, septation and swelling but decreased hydrophobicity. Intracellular activities of esterase and of mitochondria were lowered and the mitochondria became randomly organized within the cell and changed their morphology^{42, 99}. In general, comparable responses were recorded for *P. ultimum*, which in addition changed the morphology of nuclei in response to viscosinamide. The antifungal activity of the viscosin-group CLPs pseudophomins A and B has been tested against several phytopathogenic fungi grown on solid media. These compounds showed a relatively weak effect on *R. solani*, whereas in particular pseudophomin B was able to cause a significant growth inhibition for *Phoma lingam*, *Alternaria brassicae* and *Sclerotinia sclerotiorum*⁷⁷.

Amphisin-group CLPs show a stronger antagonistic effect to *P. ultimum* and *R. solani* than viscosinamide⁷⁴, and for amphisin an MIC of 10 µg/ml (c. 7 µM) has been determined for cultures growing on solid media². Hyphal growth of *R. solani* challenged with tensin has been studied by microscopy. Tensin-challenged hyphae typically became hyaline and swollen, and branching of the mycelium increased close to the tensin source⁷³. Interestingly, growth of *R. solani* in liquid media amended with viscosinamide or tensin was not negatively affected, although increased branching and darkening of the medium was observed for the tensin-challenged cultures.

Among the large hydrophobic CLPs, tolaasin inhibits growth of a large number of fungal cultures⁸², but the toxin has been particularly well studied due to its role in the development of the brown blotch disease of *A. bisporus* caused by *P. tolaasii* and related strains⁹². During the cause of this disease dark, sunken spots develop on the surface of the mushroom. These symptoms can be mimicked by tolaasin at concentration of 10 µg/ml (browning) and 30 µg/ml (pitting) during a 16-hr incubation⁹². Tolaasin preparations increase tyrosinase activity of the mushroom, and the ability of the enzyme to oxidize phenols leads to discolouration (browning). A direct activation of the tyrosinases is possibly due to the action of proteinases that are released from the fungal tissues as a consequence of cell disruption caused by tolaasin. Additionally, induction of specific tyrosinase mRNAs has been demonstrated

upon treatment with purified tolaasin. It is, however, unclear if this is a direct effect of tolaasin or an indirect result of membrane disruption⁹³.

Studies addressing the impact of syringopeptins on fungal growth have frequently employed *Geotrichum candidum*, *Botrytis cinerea* and the yeast *Rhodotorula pilimanae* as model organisms⁵⁶. The syringopeptins have variable effects on these fungi. Hence syringopeptin 22A is a more powerful antimicrobial compound for *R. pilimanae* and *G. candidum* than syringopeptin 25A, whereas the reverse is true for *B. cinerea*. For comparison, syringopeptin PhvA inhibits neither *R. pilimanae* nor *G. candidum* at concentrations as high as 50 μM ³⁶, and the effect of corpeptin against *R. pilimanae* is negligible²⁶.

Syringopeptins inhibit the germination of fungal spores or conidia at considerable lower concentrations than those that inhibit growth. For example 0.6 μM syringopeptin 22A and syringopeptin 25A inhibit germination of *G. candidum* conidia by 78% and 60%, respectively, while growth of the fungus is inhibited at 12.5 and 1.6 μM , respectively⁵⁶. Synergistic actions between syringopeptin 25A and cell wall-degrading enzymes as endochitinase and glucanase have been demonstrated for several fungi using spore germination assays^{29, 103}. Typically the 50% effective dose of the CLP was increased by at least tenfold, so that in the presence of hydrolytic enzymes the ED_{50} was as low as 0.03 $\mu\text{g/ml}$.

Syngomycin E and syngotoxin inhibit growth of several fungi^{14, 56} but there is no consistent difference between the MIC values obtained for these compounds (6.25–37.5 μM) and those obtained for syringopeptins 22A or 25A. Sørensen and co-workers⁹⁴ reported that syngomycin E, syngotoxin B and syngostatin A were all more effective against yeasts than against filamentous fungi, and found that syngotoxin was a less active antifungal compound (0.8–200 $\mu\text{g/ml}$) than syngomycin and syngostatin (2.5–40 $\mu\text{g/ml}$). Later studies suggest a comparable toxicity of syngomycin E to *Aspergillus* and *Fusarium* species that are killed by concentrations below 7.8 $\mu\text{g/ml}$ (c. 7 μM)²¹.

The ecomycins inhibit growth of a large number of human and plant pathogens⁶¹. *Cryptococcus neoformans* and *Candida glabrans* were the most sensitive organisms as MIC values for ecomycin B were 4–8 $\mu\text{g/ml}$. Finally pseudomycin B is highly active against the human pathogens *Candida albicans* and *C. neoformans* at 0.6 and 0.01 $\mu\text{g/ml}$ ¹⁰⁵, and pseudomycin A appears to be active against *Ophiostoma ulmi* causing Dutch elm disease, see Coiro *et al.*¹⁸.

Synergistic relations have also been observed between cell wall-degrading enzymes and syngomycin E. This CLP is generally a more effective inhibitor of spore germination than syringopeptin 25A when tested without hydrolytic enzymes^{29, 103}, whereas the reverse is true in the presence of hydrolytic enzymes. The higher activity of syringopeptin than of syngomycins on target

cells with a degraded cell wall is in agreement with the higher ability to disrupt (form channels) in different types of artificial lipid membranes^{1, 17}, and with the haemolytic properties of these CLPs^{1, 20}.

5.3.4. *Structure–Function Relations for CLPs and Perspectives for Future Exploitation as Antibiotics*

The antibiotic properties of relatively few *Pseudomonas* CLPs have been tested against larger panels of target organisms. Nevertheless certain patterns emerge for the scattered information that is available: Among bacteria, mycobacteria have a high sensitivity to members of several CLP groups, with syringopeptin and syringomycin being most potent compounds tested so far. Syringopeptin is a potent antibiotic for at least some other Gram-positives, while the effects of syringomycin, syringotoxin and in particular viscosin are less severe. Antifungal activities are associated with CLPs from all four major groups, yet it is difficult to draw more specific conclusions from the experimental data than that the anti-fungal activity of the CLPs depends both on the compound and on the fungal model organisms⁵⁶. Hence, quite few specific structure–function relationships have been revealed. For tolaasin, three amino acids from the α -helical part of the molecule are essential for pore-formation, lysis of erythrocytes and induction of disease symptoms on mushroom tissues¹², and for syringomycin, the Thr-4-Cl residue is required for toxicity¹⁰.

Pseudomonas CLPs or their derivatives may have a potential as anti-mycobacterial and/or anti-fungal drugs. For example, pseudomycin B is effective against pathogenic fungi in vivo in mice and rats, but causes irritation and eventually necrosis of the affected tissues. Interestingly, pseudomycin analogues have been synthesized, which retain antifungal effects in vitro and in vivo, but show less toxicity to experimental animals¹⁰⁵. Even the ecomycins that are active against important human fungal pathogens lack toxicity to humans⁶¹.

6. SIGNIFICANCE OF *PSEUDOMONAS* CLP PRODUCTION IN THE ENVIRONMENT

Production of CLPs by pseudomonads and the stability of the compounds in (more or less) natural environments have only rarely been documented. In a recent study, Nielsen and Sørensen⁷⁵ found that amphisin and tensin were produced in the rhizosphere of young sugar beet plants, while viscosinamide was maintained at a relatively high level in this habitat. In contrast, these CLPs were not produced in a bulk soil environment. Once produced, the CLPs are not immediately degraded. For example, the introduction of purified

viscosinamide, tensin and amphisin to soil demonstrated that the compounds persisted for a few weeks before they were degraded by indigenous micro-organisms⁷⁵. Considering these data and the above-mentioned significance of plant signal molecules for expression of several CLPs, it is likely that CLPs are mainly produced in specific habitats, for example, at plant surfaces. As summarized below, there are also some reports that address the possible functions of *Pseudomonas* CLPs in these and other natural habitats.

The production of CLP biosurfactants that increase the wettability of surfaces is likely to improve the solubility of hydrophobic substrates, for example, plant wax layers⁴⁶ or nutrients, that the CLP-producing organisms, and possibly other organisms as well, can utilize for growth⁵⁹. If specific uptake mechanisms for hydrophobic substrates are involved they could possibly be analogous to the system found in *P. aeruginosa*, where rhamnolipid biosurfactants stimulate fast uptake of hydrophobic compounds as hexadecane⁷⁶.

Pseudomonas CLPs can solubilize plant wax layers, which may facilitate the action of cell wall-degrading enzymes involved in virulence. This has been demonstrated in vitro by incubating broccoli florets in a mixture of pectolytic enzymes and viscosin. The mixture caused decay of broccoli florets, while an enzyme solution alone could not cause this effect⁴⁶. Hence, a more general function of these surfactants might be to improve the access of hydrolytic enzymes to polymeric substrates as proteins or carbohydrates that are normally protected by hydrophobic surface layers.

There is a considerable interest to use biosurfactants as alternatives to chemical surfactants, for example, in the oil, food and cosmetic industries, in part due their higher biodegradability and lower toxicity^{7, 55}. The major "environmental" applications so far are in oil production, oil recovery and in oil spill bioremediation⁷. Biosurfactants further play an important role in the degradation of other hydrophobic pollutants as polyaromatic hydrocarbons (PAH). The surfactants solubilize the pollutants, thereby enhancing their degradation in the environment. Even heavy metals can be removed from polluted soil or sediments by batch washing treatments with biosurfactants⁶⁸. This application makes use of the ability of some surfactants to chelate metal ions. Hence, the biosurfactants can chelate metal ions adsorbed to soil solids, and subsequently the metal-surfactant complex in the soil solution can be removed by washing. The biosurfactants that have been useful in bioremediation experiments are often not fully characterized, but surfactin from *B. subtilis*, sophorolipids from the yeast *Candida (Torulopsis) bombicola* and rhamnolipids produced by *P. aeruginosa* have attracted much attention^{7, 55}. Possibly, CLPs from *Pseudomonas* could be used for bioremediation as well, but this possibility has not been explored so far.

Plant-associated pseudomonads may use the amphiphilic CLPs to condition their cell surfaces as well as their substratum so as to enable surface

motility on, for example, leaf surfaces. For example, a viscosin-producing pectolytic *P. fluorescens* strain could colonize and spread on hydrophobic broccoli floret surfaces, where it caused broccoli head rot⁴⁶. In contrast, a viscosin-deficient Tn5 mutant strain was unable to colonize the surface and to spread on the florets, and viscosin could complement the mutant phenotype. A more general observation along the same line, but not specifically involving pseudomonads, is that many bacteria living on surfaces secrete wetting agents (or biosurfactants), and that mutants defective in production of these agents have a lower accessibility to hydrophobic surfaces and are impaired in spreading across these surfaces⁶⁰.

A hand-full of studies support the notion that CLP-producing *Pseudomonas* strains can not only inhibit fungi under in vitro conditions but actually be useful for biological control of pre-emergence damping-off pathogens or post-harvest fungal pathogens. The viscosinamide-producer *P. fluorescens* strain DR54, which is antagonistic to *P. ultimum* and *R. solani* in vitro has been introduced to soil-plant microcosms to determine root colonization, metabolite production and antagonisms against the above fungi^{100, 101}. DR54 proved to be a good colonizer of sugar beet roots, and produced viscosinamide on the seeds and in the rhizosphere. Furthermore the strain inhibited the growth of both fungi and induced morphological changes that resembled those demonstrated for viscosinamide in vitro^{72, 99, 100, 101}. Furthermore, *P. fluorescens* DR54 could reduce damping-off symptoms induced by *R. solani* in pot experiments and increase plant emergence in field experiments. These results point to a role for viscosinamide in antagonism in vivo, but it has not been established whether viscosin acts alone, or in synergism with other antibiotics or exo-enzymes.

Syringomycin-producing *P. syringae* strains are commercially available to control post-harvest diseases, for example, on lemons¹⁴. Although the strains inhibit formation of green mold caused by *Penicillium digitatum*, the concentration of purified syringomycin needed to inhibit the disease was about 1,000 times the concentration needed to inhibit *P. digitatum* in vitro. This discrepancy could reflect the need of synergistic actions between syringomycin and other antagonistic compounds for effective disease control. Biocontrol synergism has been reported for another *P. syringae* strain and the biocontrol fungus *Trichoderma atroviride* strain P1²⁹ after simultaneous application of these two strains to control *B. cinerea* on artificially infected apples. Subsequent experiments including an endochitinase-negative mutant of *T. atroviride* as well as a chitinase inhibitor demonstrated that this enzyme played a role in the synergism, which is in agreement with the synergism between CLPs and cell wall-degrading enzymes demonstrated in vitro^{29, 103}.

The synergistic interactions between hydrolytic enzymes and syringomycin or syringopeptin, which are required for efficient biocontrol of

fungus pathogens resemble the interactions between pectolytic enzymes and viscosin that are necessary for development of broccoli head rot. On top of this, the synergistic effects of CLP-mediated surface motility and unknown antifungal compounds for in vitro biocontrol effect may play a role in the antagonism between pseudomonads and other microorganisms in natural environments. Hence, we speculate that studies on the interactions between CLPs and other cellular functions may provide the key to a future understanding of the biological significance by CLP-production for pseudomonads in natural habitats. In addition, if *Pseudomonas* CLPs can chelate metal ions, they might also be essential for the acquisition of micronutrients in habitats conducive for CLP production.

REFERENCES

1. Agner, G., Kaulin, Y.A., Gurnev, P.A., Szabo, Z., Schagina, L.V., Takemoto, J.Y., and Blasko, K., 2000, Membrane-permeabilizing activities of cyclic lipodepsipeptides, syringopeptin 22A and syringomycin E from *Pseudomonas syringae* pv. *syringae* in human blood cells and in bilayer lipid membranes. *Bioelectrochemistry*, 52:161–167.
2. Andersen, J.B., Koch, B., Nielsen, T.H., Sørensen, D., Hansen, M., Nybroe, O., Christophersen, C., Sørensen, J., Molin, S., and Givskov, M., 2003, Surface motility in *Pseudomonas* sp. DSS73 is required for efficient biological containment of the root-pathogenic microfungi *Rhizoctonia solani* and *Pythium ultimum*. *Microbiology-SGM*, 149:37–46.
3. Ballio, A., Bossa, F., Di Giorgio, D., Ferranti, P., Paci, M., Pucci, P., Scaloni, A., Segre, A., and Strobel, G.A., 1994, Novel bioactive lipodepsipeptides from *Pseudomonas syringae*—The pseudomycins. *FEBS Lett.*, 355:96–100.
4. Ballio, A., Collina, A., Di Nola, A., Manetti, C., Paci, M., and Segre, A., 1994, Determination of structure and conformation in solution of syringotoxin, a lipodepsipeptide from *Pseudomonas syringae* pv. *syringae* by 2D NMR and molecular dynamics. *Struct. Chem.*, 5:43–50.
5. Ballio, A., Bossa, F., Di Giorgio, D., Di Nola, A., Manetti, C., Paci, M., Scaloni, A., and Segre, A.L., 1995, Solution conformation of the *Pseudomonas syringae* pv. *syringae* phytotoxic lipodepsipeptide syringopeptin 25-A. Two-dimensional NMR, distance geometry and molecular dynamics. *Eur. J. Biochem.*, 234:747–758.
6. Ballio, A., Bossa, F., Camoni, L., Di Giorgio, D., Flamand, M.-C., Maraite, H., Nitti, G., Pucci, P., and Scaloni, A., 1996, Structure of fuscopeptins, phytotoxic metabolites of *Pseudomonas fuscovaginae*. *FEBS Lett.*, 381:213–216.
7. Banat, I.M., Makkar, R.S., and Cameotra, S.S., 2000, Potential commercial applications of microbial surfactants. *Appl. Microbiol. Biotechnol.*, 53:495–508.
8. Baré, S., Coiro, V.M., Scaloni, A., Di Nola, A., Paci, M., Segre, A.L., and Ballio, A., 1999, Conformation in solution of the fuscopeptins. *Eur. J. Biochem.*, 266:484–492.
9. Batoko, H., de Kerchove d'Exaerde, A., Kinet, J.-M., Bouharmont, J., Gage, R.A., Maraite, H., and Boutry, M., 1998, Modulation of plant plasma membrane H⁺-ATPase by phytotoxic lipodepsipeptides produced by the plant pathogen *Pseudomonas fuscovaginae*. *Biochim. Biophys. Acta*, 1372:216–226.
10. Bender, C.L., Alarcón-Chaidez, F., and Gross, D.C., 1999, *Pseudomonas syringae* phytotoxins: Mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. *Microbiol. Mol. Biol. Rev.*, 63:266–292.

11. Braun, P.G., Hildebrand, P.D., Ells, T.C., and Kobayashi, D.Y., 2001, Evidence and characterization of a gene cluster required for the production of viscosin, a lipopeptide biosurfactant, by a strain of *Pseudomonas fluorescens*. *Can. J. Microbiol.*, 47:294–301.
12. Brodey, C.L., Rainey, P.B., Tester, M., and Johnstone, K., 1991, Bacterial blotch disease of the cultivated mushroom is caused by an ion channel forming lipodepsipeptide toxin. *Mol. Plant-Microbe Interact.*, 4:407–411.
13. Buber, E., Stindl, A., Acan, N.L., Kocagoz, T., and Zocher, R., 2002, Antimycobacterial activity of lipodepsipeptides produced by *Pseudomonas syringae* pv. *syringae* B359. *Nat. Prod. Lett.*, 16:419–423.
14. Bull, C.T., Wadsworth, M.L., Sorensen, K.N., Takemoto, J.Y., Austin, R.K., and Smilanick, J.L., 1998, Syringomycin E produced by biological control agents controls green mold on lemons. *Biol. Control*, 12:89–95.
15. Bull, C.T., Duffy, B., Voisard, C., Defago, G., Keel, C., and Haas, D., 2001, Characterization of spontaneous *gacS* and *gacA* regulatory mutants of *Pseudomonas fluorescens* biocontrol strain CHA0. *Antonie van Leeuwenhoek*, 79:327–336.
16. Bultreus, A. and Gheysen, I., 1999, Biological and molecular detection of toxic lipodepsipeptide-producing *Pseudomonas syringae* strains and PCR identification in plants. *Appl. Environ. Microbiol.*, 65:1904–1909.
17. Camoni, L., Di Giorgio, D., Marra, M., Aducci, P., and Ballio, A., 1995, *Pseudomonas syringae* pv. *syringae* phytotoxins reversibly inhibit the plasma membrane H^+ -ATPase and disrupt unilamellar liposomes. *Biochem. Biophys. Res. Comm.*, 214:118–124.
18. Coiro, V.M., Segra, A.L., Di Nola, A., Paci, M., Grottesi, A., Veglia, G., and Ballio, A., 1998, Solution conformation of the *Pseudomonas syringae* MSU16H phytotoxic lipodepsipeptide pseudomycin A determined by computer simulations using distance geometry and molecular dynamics from NMR data. *Eur. J. Biochem.*, 257:449–456.
19. Dalla Serra, M., Bernhart, I., Nordera, P., Di Giorgio, D., Ballio, A., and Menestrina, G., 1999, Conductive properties and gating of channels formed by syringopeptin 25A, a bioactive lipodepsipeptide from *Pseudomonas syringae* pv. *syringae*, in planar lipid membranes. *Mol. Plant-Microbe Interact.*, 12:401–409.
20. Dalla Serra, M., Fagioli, G., Nordera, P., Bernhart, I., Della Volpe, C., Di Giorgio, D., Ballio, A., and Menestrina, G., 1999, The interaction of lipodepsipeptide toxins from *Pseudomonas syringae* pv. *syringae* with biological and model membranes: A comparison of syringotoxins, syringomycins, and two syringopeptins. *Mol. Plant-Microbe Interact.*, 12:391–400.
21. De Lucca, A.J., Jacks, T.J., Takemoto, J., Vinyard, B., Peter, J., Navarro, E., and Walsh, T.J., 1999, Fungal lethality, binding and cytotoxicity of syringomycin-E. *Antimicrob. Agents Chemother.*, 43:371–373.
22. Di Giorgio, D., Camoni, L., Marchiafava, C., and Ballio, A., 1997, Biological activities of pseudomycin A, a lipodepsinona peptide from *Pseudomonas syringae* MSU16H. *Phytochemistry*, 45:1385–1391.
23. Doekel, S. and Marahel, M.A., 2001, Biosynthesis of natural products on modular peptide synthetases. *Metabol. Eng.*, 3:64–77.
24. Dowling, D.N. and O’Gara, F., 1994, Metabolites of *Pseudomonas* involved in the biocontrol of plant disease. *TIBTECH*, 12:133–141.
25. El Sayed, K.A., Bartyzel, P., Shen, X., Perry, T.L., Zjawiony, J.K., and Hamann, M.T., 2000, Marine natural products as antituberculosis agents. *Tetrahedron*, 56:949–953.
26. Emanuele, M.C., Scaloni, A., Lavermicocca, P., Iacobellis, N.S., Camoni, L., Di Giorgio, D., Pucci, P., Paci, M., Segre, A., and Ballio, A., 1998, Corpeptins, new bioactive lipodepsipeptides from cultures of *Pseudomonas corrugata*. *FEBS Lett.*, 433:317–320.
27. Flamand, M.-C., Pellser, S., Ewbank, E., and Maraite, H., 1996, Production of syringotoxin and other bioactive peptides by *Pseudomonas fuscovaginae*. *Physiol. Mol. Plant Pathol.*, 48:217–231.

28. Fogliano, V., Gallo, M., Vinale, F., Ritieni, A., Randazzo, G., Greco, M., Lops, R., and Graniti, A., 1999, Immunological detection of syringopeptins produced by *Pseudomonas syringae* pv. lachrymans. *Physiol. Mol. Plant Pathol.*, 55:255–261.
29. Fogliano, V., Ballio, A., Gallo, M., Woo, S., Scala, F., and Lorito, M., 2002, *Pseudomonas* lipodepsipeptides and fungal cell wall-degrading enzymes act synergistically in biological control. *Mol. Plant-Microbe Interact.*, 15:323–333.
30. Fukuchi, N., Isogai, A., Nakayama, J., Takayama, S., Yamashita, S., Suyama, K., and Suzuki, A., 1992, Structure and stereochemistry of three phytotoxins, syringomycin, syringotoxin and syringostatin produced by *Pseudomonas syringae* pv. *syringae*. *J. Chem. Soc. Perkin Trans. I*, 1149–1157.
31. Gerard, J., Lloyd, R., Barsby, T., Haden, P., Kelly, M.T., and Andersen, R.J., 1997, Massetolids A-H, Antimycobacterial cyclic depsipeptides produced by two pseudomonads isolated from marine habitats. *J. Nat. Prod.*, 60:223–229.
32. Godfrey, S.A.C., Marshall, J.W., and Klena, J.D., 2001, Genetic characterization of *Pseudomonas* 'NZ17'—a novel pathogen that results in a brown blotch disease of *Agaricus bisporus*. *J. Appl. Microbiol.*, 91:412–420.
33. Grangemard, I., Wallach, J., Maget-Dana, R., and Peypoux, F., 2001, Lichenysin—A more efficient cation chelator than surfactin. *Appl. Biochem. Biotech.*, 90:199–210.
34. Grewal, S.I.S., Han, B., and Johnstone, K., 1995, Identification and characterization of a locus which regulates multiple functions in *Pseudomonas tolaasii*, the cause of brown blotch disease of *Agaricus bisporus*. *J. Bacteriol.*, 177:4658–4668.
35. Grgurina, I., Gross, D.C., Iacobellis, N.S., Lavermicocca, P., Takemoto, J.Y., and Benincasa, M., 1996, Phytotoxin production by *Pseudomonas syringae* pv. *syringae*: Syringopeptin production by *syr* mutants defective in biosynthesis or secretion of syringomycin. *FEMS Microbiol. Lett.*, 138:35–39.
36. Grgurina, I., Mariotti, F., Fogliano, V., Gallo, M., Scaloni, A., Iacobellis, N.S., Lo Cantore, P., Mannina, L., van Axel Castelli, V., Grego, M.L., and Graniti, A., 2002, A new syringopeptin produced by bean strains of *Pseudomonas syringae* pv. *syringae*. *Biochim. Biophys. Acta*, 1597:81–89.
37. Gross, D.C., 1985, Regulation of syringomycin synthesis in *Pseudomonas syringae* pv. *syringae* and defined conditions for its production. *J. Appl. Bacteriol.*, 58:167–174.
38. Groupé, V., Pugh, L.H., Weiss, D., and Kochi, M., 1951, Observations on the antiviral activity of viscosin. *P. Soc. Exp. Biol. Med.*, 78:354–358.
39. Guenzi, E., Galli, G., Grgurina, I., Gross, D.C., and Grandi, G., 1998, Characterization of the syringomycin synthetase gene cluster. *J. Biol. Chem.*, 273:32857–32863.
40. Han, B., Pain, A., and Johnstone, K., 1997, Spontaneous duplication of a 661 bp element within a two-component sensor regulator gene causes phenotypic switching in colonies of *Pseudomonas tolaasii*, cause of brown blotch disease of mushrooms. *Mol. Microbiol.*, 25:211–218.
41. Han, F., Mortishire-Smith, R.J., Rainey, P.B., and Williams, D.H., 1992, Structure of the white-line inducing principle isolated from *Pseudomonas reactans*. *Acta Cryst.*, C48:1965–1968.
42. Hansen, M., Thrane, C., Olsson, S., and Sørensen, J., 2000, Confocal imaging of living fungal hyphae challenged with the fungal antagonist viscosinamide. *Mycologia*, 92:216–221.
43. Heeb, S. and Haas, D., 2001, Regulatory roles of the GacS/GacA two-component system in plant-associated and other Gram-negative bacteria. *Mol. Plant-Microbe Interact.*, 14:1351–1363.
44. Henriksen, A., Anthoni, U., Nielsen, T.H., Sørensen, J., Christophersen, C., and Gajhede, M., 2000, Cyclic lipoundecapeptide tensin from *Pseudomonas fluorescens* strain 96.578. *Acta Cryst.*, C56:113–115.
45. Hildebrand, P.D., 1989, Surfactant-like characteristics and identity of bacteria associated with broccoli head rot in Atlantic Canada. *Can. J. Plant Path.*, 11:205–214.

46. Hildebrand, P.D., Braun, P.G., McRae, K.B., and Lu, X., 1998, Role of the biosurfactant viscosin in broccoli head rot caused by a pectolytic strain of *Pseudomonas fluorescens*. *Can. J. Plant. Pathol.*, 20:296–303.
47. Hrabak, E.M. and Willis, D.K., 1992, The *lemA* gene required for pathogenicity of *Pseudomonas syringae* pv. *syringae* on bean is a member of a family of two-component regulators. *J. Bacteriol.*, 174:3011–3020.
48. Hu, F.-P., Young, J.M., and Fletcher, M.J., 1998, Preliminary description of biocidal (syringomycin) activity in fluorescent plant pathogenic *Pseudomonas* species. *J. Appl. Microbiol.*, 85:357–364.
49. Hutchison, M.L., Tester, M.A., and Gross, D.C., 1995, Role of biosurfactant and ion-channel-forming activities of syringomycin in transmembrane ion flux—a model for the mechanism of action in the plant-pathogen interaction. *Mol. Plant-Microbe Interact.*, 8:610–620.
50. Hutchison, M.L. and Gross, D.C., 1997, Lipopeptide phytotoxins produced by *Pseudomonas syringae* pv. *syringae*: Comparison of the biosurfactant and ion channel-forming activities of syringopeptin and syringomycin. *Mol. Plant-Microbe Interact.*, 10:347–354.
51. Ivanova, E.P., Gorshkova, N.M., Sawabe, T., Hayashi, K., Kalinovskaya, N.I., Lysenko, A.M., Zhukova, N.V., Nicolau, D.V., Kuznetsova, T.A., Mikhailov, V.V., and Christen, R., 2002, *Pseudomonas extremorientalis* sp. nov., isolated from a drinking water reservoir. *Int. J. Syst. Evol. Microbiol.*, 52:2113–2130.
52. Keel, C., Weller, D.M., Natsch, A., Défago, G., Cook, R.J., and Thomashow, L.S., 1996, Conservation of the 2,4-diacetylphloroglucinol biosynthesis locus among fluorescent *Pseudomonas* strains from diverse geographic locations. *Appl. Environ. Microbiol.*, 62:552–563.
53. Koch, B., Nielsen, T.H., Sørensen, D., Andersen, J.B., Christophersen, C., Molin, S., Givskov, M., Sørensen, J., and Nybroe, O., 2002, Lipopeptide production in *Pseudomonas* sp. strain DSS73 is regulated by components of sugar beet seed exudates via the Gac two-component regulatory system. *Appl. Environ. Microbiol.*, 68:4509–4516.
54. Konz, D. and Marahiel, M.A., 1999, How do peptide synthetases generate structural diversity. *Chem. Biol.*, 6:R39–R48.
55. Lang, S., 2002, Biological amphiphiles (microbial biosurfactants). *Curr. Opin. Coll. Interface Sci.*, 7:12–20.
56. Lavermicocca, P., Sante Iacobellis, N., Simmaco, M., and Graniti, A., 1997, Biological properties and spectrum of activity of *Pseudomonas syringae* pv. *syringae* toxins. *Physiol. Mol. Plant Pathol.*, 50:129–140.
57. Laycock, M.V., Hildebrand, P.D., Thibault, P., Walter, J.A., and Wright, J.L.C., 1991, Viscosin, a potent peptidolipid biosurfactant and phytopathogenic mediator produced by a pectolytic strain of *Pseudomonas fluorescens*. *J. Agric. Food Chem.*, 39:483–489.
58. Lee, H.-I., Jeong, K.-S., and Cha, J.-S., 2002, PCR assays for specific and sensitive detection of *Pseudomonas tolaasii*, the cause of brown blotch disease of mushrooms. *Lett. Appl. Microbiol.*, 35:276–280.
59. Lindow, S.E. and Brandl, M.T., 2003, Microbiology of the phyllosphere. *Appl. Environ. Microbiol.*, 69:1875–1883.
60. Matsuyama, T. and Nakagawa, Y., 1996, Bacterial wetting agents working in colonization of bacteria on surface environments. *Coll. Surf. B*, 7:207–214.
61. Miller, C.M., Miller, R.V., Garton-Kenny, D., Redgrave, B., Sears, J., Condrón, M.M., Teplow, D.B., and Strobel, G.A., 1998, Ecomycins, unique antimycotics from *Pseudomonas viridiflava*. *J. Appl. Microbiol.*, 84:937–944.
62. Mo, Y.-Y. and Gross, D.C., 1991, Plant signal molecules activate the *syrB* gene, which is required for syringomycin production by *Pseudomonas syringae* pv. *syringae*. *J. Bacteriol.*, 173:5784–5792.

63. Mo, Y.-Y., Geibel, M., Bonsall, R.F., and Gross, D.C., 1995, Analysis of sweet cherry (*Prunus avium* L.) leaves for plant signal molecules that activate the *syfB* gene required for synthesis of the phytotoxin, syringomycin, by *Pseudomonas syringae* pv. *syringae*. *Plant Physiol.*, 107:603–612.
64. Monti, S.M., Gallo, M., Ferracane, R., Borrelli, R.C., Ritieni, A., Greco, M.L., Graniti, A., and Fogliano, V., 2001, Analysis of bacterial lipopeptides by matrix-assisted laser desorption/ionisation time-of-flight and high-performance liquid chromatography with electrospray mass spectrometry. *Rapid Commun. Mass Spectrom.*, 15:623–628.
65. Morikawa, M., Hirata, Y., and Imanaka, T., 2000, A study of the structure–function relationship of lipopeptide surfactants. *Biochim. Biophys. Acta*, 1488:211–218.
66. Mortishire-Smith, R.J., Drake, A.F., Nutkins, J.C., and Williams, D.H., 1991, Left-handed α -helix formation by a bacterial peptide. *FEBS Lett.*, 278:244–246.
67. Mortishire-Smith, R.J., Nutkins, J.C., Packman, L.C., Brodey, C.L., Rainey, P.B., Johnstone, K., and Williams, D.H., 1991, Determination of the structure of an extracellular peptide produced by the mushroom pathogen saprotroph *Pseudomonas reactans*. *Tetrahedron*, 47:3645–3654.
68. Mulligan, C.N., Young, R.N., and Gibbs, B.F., 2001, Heavy metal removal from sediments by biosurfactants. *J. Hazard. Mat.*, 85:111–125.
69. Munsch, P. and Alatossava, T., 2002, The white-line-in-agar test is not specific for the two cultivated mushroom associated pseudomonads, *Pseudomonas tolaasii* and *Pseudomonas 'reactans'*. *Microbiol. Res.*, 157:7–11.
70. Munsch, P., Alatossava, T., Marttinen, N., Meyer, J.-M., Christen, R., and Gardan, L., 2002, *Pseudomonas constantinii* sp. nov., another causal agent of brown blotch disease, isolated from cultivated mushroom sporophores in Finland. *Int. J. Syst. Evol. Microbiol.*, 52:1973–1983.
71. Nielsen, M.N., Sørensen, J., Fels, J., and Pedersen, H.C., 1998, Secondary metabolite- and endochitinase-dependent antagonism toward plant-pathogenic microfungi of *Pseudomonas fluorescens* isolates from sugar beet rhizosphere. *Appl. Environ. Microbiol.*, 64:3563–3569.
72. Nielsen, T.H., Christophersen, C., Anthoni, U., and Sørensen, J., 1999, Viscosinamide, a new cyclic depsipeptide with surfactant and antifungal properties produced by *Pseudomonas fluorescens* DR54. *J. Appl. Microbiol.*, 86:80–90.
73. Nielsen, T.H., Thrane, C., Christophersen, C., Anthoni, U., and Sørensen, J., 2000, Structure, production characteristics and fungal antagonism of tensin—a new antifungal cyclic lipopeptide from *Pseudomonas fluorescens* strain 96.578. *J. Appl. Microbiol.*, 89:992–1001.
74. Nielsen, T.H., Sørensen, D., Tobiasen, C., Andersen, J.B., Christophersen, C., Givskov, M., and Sørensen, J., 2002, Antibiotic and biosurfactant properties of cyclic lipopeptides produced by fluorescent *Pseudomonas* spp. from the sugar beet rhizosphere. *Appl. Environ. Microbiol.*, 68:3416–3423.
75. Nielsen, T.H. and Sørensen, J., 2003, Production of cyclic lipopeptides by *Pseudomonas fluorescens* strains in bulk soil and in the sugar beet rhizosphere. *Appl. Environ. Microbiol.*, 69:861–868.
76. Noordman, W.H. and Janssen, D.B., 2002, Rhamnolipid stimulates uptake of hydrophobic compounds by *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.*, 68:4502–4508.
77. Pedras, M.S.C., Ismail, N., Quail, J.W., and Boyetchko, S.M., 2003, Structure, chemistry, and biological activity of pseudophomins A and B, new cyclic lipopeptides isolated from the biocontrol bacterium *Pseudomonas fluorescens*. *Phytochemistry*, 62:1105–1114.
78. Peypoux, F., Bonmatin, J.M., and Wallach, J., 1999, Recent trends in the biochemistry of surfactin. *Appl. Microbiol. Biotechnol.*, 51:553–563.
79. Quail, J.W., Ismail, N., Pedras, M.S.C., and Boyetchko, S.M., 2002, Pseudophomins A and B, a class of cyclic lipopeptides isolated from a *Pseudomonas* species. *Acta Cryst.*, C58:268–271.

80. Quigley, N.B. and Gross, D.C., 1994, Syringomycin production among strains of *Pseudomonas syringae* pv. *syringae*: Conservation of the *syrB* and *syrD* genes and activation of phytotoxin production by plant signal molecules. *Mol. Plant-Microbe Interact.*, 7:78–90.
81. Raaijmakers, J.M., Vlami, M., and de Souza, J.T., 2002, Antibiotic production by bacterial biocontrol agents. *Antonie van Leeuwenhoek*, 81:537–547.
82. Rainey, P.B., Brodey, C.L., and Johnstone, K., 1991, Biological properties and spectrum of activity of tolaasin, a lipodepsipeptide toxin produced by the mushroom pathogen *Pseudomonas tolaasii*. *Physiol. Mol. Plant Pathol.*, 39:57–70.
83. Rainey, P.B., Brodey, C.L., and Johnstone, K., 1992, Biology of *Pseudomonas tolaasii*, cause of brown blotch disease of the cultivated mushroom. *Adv. Plant. Pathol.*, 39:105–106.
84. Rainey, P.B., Brodey, C.L., and Johnstone, K., 1993, Identification of a gene-cluster encoding 3 high-molecular-weight proteins, which is required for synthesis of tolaasin by the mushroom pathogen *Pseudomonas tolaasii*. *Mol. Microbiol.*, 8:643–652.
85. Rajendran, N., 1999, Identification and cloning of a gene locus encoding peptide synthetase of *Pseudomonas fluorescens* by two sets of PCR primers. *Z. Naturforsch.*, 54c:105–109.
86. Rautenbach, M., Swart, P., and van der Merwe, M.J., 2000, The interaction of analogues of the antimicrobial lipopeptide iturin A(2) with alkali metal ions. *Bioorg. Med. Chem.*, 8:2539–2548.
87. Scholz-Schroeder, B.K., Hutchison, M.L., Grgurina, I., and Gross, D.C., 2001, The contribution of syringopeptin and syringomycin to virulence of *Pseudomonas syringae* pv. *syringae* strain B301D on the basis of *sypA* and *syrB1* biosynthesis mutants. *Mol. Plant-Microbe Interact.*, 14:336–348.
88. Scholz-Schroeder, B.K., Soule, J.D., Lu, S.E., Grgurina, I., and Gross, D.C., 2001, A physical map of the syringomycins and syringopeptin gene clusters localized to an approximately 145-kb DNA region of *Pseudomonas syringae* pv. *syringae* strain B301D. *Mol. Plant-Microbe Interact.*, 14:1426–1435.
89. Scholz-Schroeder, B.K., Soule, J.D., and Gross, D.C., 2003, The *sypA*, *sypB* and *sypC* synthetase genes encode twenty-two modules involved in the nonribosomal peptide synthesis of syringopeptin by *Pseudomonas syringae* pv. *syringae* B301D. *Mol. Plant-Microbe Interact.*, 16:271–280.
90. Segre, A., Bachman, R.C., Ballio, A., Bossa, F., Grgurina, I., Iacobellis, N.S., Marino, G., Pucci, P., Simmaco, M., and Takemoto, J.Y., 1989, The structure of syringomycin-A1, syringomycin-E and syringomycin-G. *FEBS Lett.*, 255:27–31.
91. Soler-Rivas, C., Arpin, N., Olivier, J.M., and Wichers, H.J., 1999, WLIP, a lipodepsipeptide of *Pseudomonas reactans*, as inhibitor of the symptoms of the brown blotch disease of *Agaricus bisporus*. *J. Appl. Microbiol.*, 86:635–641.
92. Soler-Rivas, C., Jolivet, S., Arpin, N., Olivier, J.M., and Wichers, H.J., 1999, Biochemical and physiological aspects of brown blotch disease of *Agaricus bisporus*. *FEMS Microbiol. Rev.*, 23:591–614.
93. Soler-Rivas, C., Möller, A.C., Arpin, N., Olivier, J.-M., and Wichers, H.J., 2001, Induction of a tyrosinase mRNA in *Agaricus bisporus* upon treatment with a tolaasin preparation from *Pseudomonas tolaasii*. *Physiol. Mol. Plant. Pathol.*, 58:95–99.
94. Sorensen, K.N., Kim, K.H., and Takemoto, J.Y., 1996, In vitro antifungal and fungicidal activities and erythrocyte toxicities of cyclic lipodepsinonapeptides produced by *Pseudomonas syringae* pv. *syringae*. *Antimicrob. Agents Chemother.*, 40:2710–2713.
95. Sorensen, K.N., Kim, K.-H., and Takemoto, J.Y., 1998, PCR detection of cyclic lipodepsinonapeptide-producing *Pseudomonas syringae* pv. *syringae* and similarity of strains. *Appl. Environ. Microbiol.*, 64:226–230.
96. Sørensen, D., Nielsen, T.H., Christophersen, C., Sørensen, J., and Gajhede, M., 2001, Cyclic lipoundecapeptide amphisin from *Pseudomonas* sp. strain DSS73. *Acta Cryst.*, C57:1123–1124.

97. Sørensen, D., 2002, Cyclic lipopeptides from *Pseudomonas* spp. Study and characterization of novel antibiotics and biocontrol agents. Ph.D. thesis. Department of Chemistry, University of Copenhagen.
98. Sørensen, D., Nielsen, T.H., Sørensen, J., and Christophersen, C., 2002, Cyclic lipoundecapeptide lokisin from *Pseudomonas* sp. DSS41. *Tetrahedron Lett.*, 43:4421–4423.
99. Thrane, C., Olsson, S., Nielsen, T.H., and Sørensen, J., 1999, Vital fluorescent stains for detection of stress in *Pythium ultimum* and *Rhizoctonia solani* challenged with viscosinamide from *Pseudomonas fluorescens* DR54. *FEMS Microbiol. Ecol.*, 30:11–23.
100. Thrane, C., Nielsen, T.H., Nielsen, M.N., Sørensen, J., and Olsson, S., 2000, Viscosinamide-producing *Pseudomonas fluorescens* DR54 exerts a biocontrol effect on *Pythium ultimum* in sugar beet rhizosphere. *FEMS Microbiol. Ecol.*, 33:139–146.
101. Thrane, C., Nielsen, M.N., Sørensen, J., and Olsson, S., 2001, *Pseudomonas fluorescens* DR54 reduces sclerotia formation, biomass development, and disease incidence of *Rhizoctonia solani* causing damping-off in sugar beet. *Microb. Ecol.*, 42:438–445.
102. Ui, H., Miyake, T., Iinuma, H., Naganawa, H., Hattori, S., Hamada, M., Takeuchi, T., Umezawa, S., and Umezawa, K., 1997, Pholipeptin, a novel cyclic lipoundecapeptide from *Pseudomonas fluorescens*. *J. Org. Chem.*, 62:103–108.
103. Woo, S., Fogliano, V., Scala, F., and Lorito, M., 2002, Synergism between fungal enzymes and bacterial antibiotics may enhance biocontrol. *Antonie van Leeuwenhoek*, 81:353–356.
104. Zhang, J.-H., Quigley, N.B., and Gross, D.C., 1997, Analysis of the *syrP* gene, which regulates syringomycin synthesis by *Pseudomonas syringae* pv. *syringae*. *Appl. Environ. Microbiol.*, 63:2771–2778.
105. Zhang, Y.-Z., Sun, X., Zeckner, D.J., Sachs, R.K., Current, W.L., Gidda, J., Rodriguez, M., and Chen, S.-H., 2001, Syntheses and antifungal activities of novel 3-amino bearing pseudomycin analogues. *Bioorg. Med. Chem. Lett.*, 11:903–907.

BIOSYNTHESIS OF RHAMNOLIPIDS

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1. INTRODUCTION

Pseudomonas aeruginosa is an environmental bacterium that can be isolated from many different habitats including water, soil and plants, but it is also an opportunistic human pathogen causing serious nosocomial infections⁸. Under specific environmental conditions this bacterium produces and secretes rhamnose-containing glycolipid biosurfactants. The production of rhamnolipids is characteristic of *P. aeruginosa*, no other Pseudomonad produces these molecules, and was first reported by Jarvis and Johnson in 1949¹⁹. The synthesis of these surfactants by cell-free extracts and the fact that they were secreted by bacteria in the stationary phase of growth was described nearly 40 years ago^{4, 5}. In liquid culture, *P. aeruginosa* produces primarily two forms of rhamnolipids (Figure 1): rhamnosyl- β -hydroxydecanoyl- β -hydroxydecanoate (mono-rhamnolipid) and rhamnosyl-rhamnosyl- β -hydroxydecanoyl- β -hydroxydecanoate (di-rhamnolipid)¹⁵.

The role that rhamnolipids play in *P. aeruginosa* life cycle and pathogenicity has not been completely understood, but they are known to affect the cellular outer membrane¹ and have been implicated in cell motility²¹ and in the formation of biofilm structure¹². Due to their tenso-active properties these compounds have several potential industrial and environmental applications^{22, 26}. These uses include the production of fine chemicals, the characterization of surfaces and surface coatings, use as additives for environmental remediation, and use as a biological control agent⁴⁵.

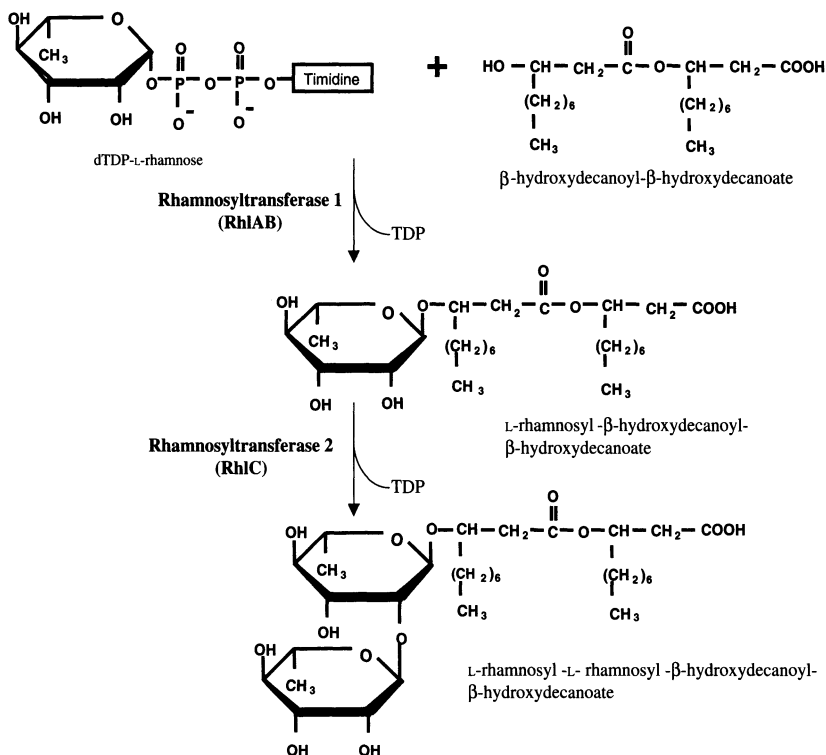


Figure 1. Diagram of rhamnolipids biosynthesis.

Here we present evidence showing that rhamnolipid production is dependent on central metabolic pathways, such as fatty acid synthesis and deoxy timidine diphosphate (dTDP)-activated sugars. We also show that the production of this biosurfactant is very tightly regulated at the transcriptional level by the quorum-sensing response and by environmental conditions; and that the production of polyhydroxyalcanoates (PHA), other biotechnological important compounds produced by *P. aeruginosa*, have some biosynthetic steps in common with their synthesis. Rhamnolipids biosynthetic pathway has also steps in common with lipopolysaccharide (LPS) and alginate synthesis.

2. ROLE OF RHAMNOSYLTRANSFERASES 1 AND 2 IN RHAMNOLIPID PRODUCTION

Pseudomonas aeruginosa cell-free extracts were found to produce rhamnolipids by two sequential reactions^{4, 5}. The first reaction is catalyzed by

rhamnosyltransferase 1 (RT1) and uses TDP-L-rhamnose and a β -hydroxy fatty acid as precursors yielding mono-rhamnolipid (Figure 1) which in turn is the substrate, together with TDP-L-rhamnose, of rhamnosyltransferase 2 (RT2) that produces di-rhamnolipid (Figure 1). RT1 is composed of two polypeptides RhlA and RhlB (Figure 2). The latter protein is the catalytic subunit³⁰ and seems to be an inner membrane protein³⁸. While RhlA role has not been determined, it is known to be very important for mono-rhamnolipid production³⁰ and seems to be loosely bound to the inner membrane³⁸. RT2 is composed of a single protein called RhlC (Figure 2) that has sequence homology with rhamnosyltransferases involved in LPS synthesis, but is specific for di-rhamnolipid synthesis³⁸. RT2 also seems to be loosely bound to the inner membrane³⁸. The subcellular localization of RT1 and RT2 suggests that rhamnolipid production and secretion are coupled (Figure 2), but the participation of other proteins in secretion has not been ruled out.

The *rhlA* and *rhlB* genes are arranged as an operon and are clustered with *rhlR* and *rhlI* (Figure 2), that encode proteins involved in their transcriptional

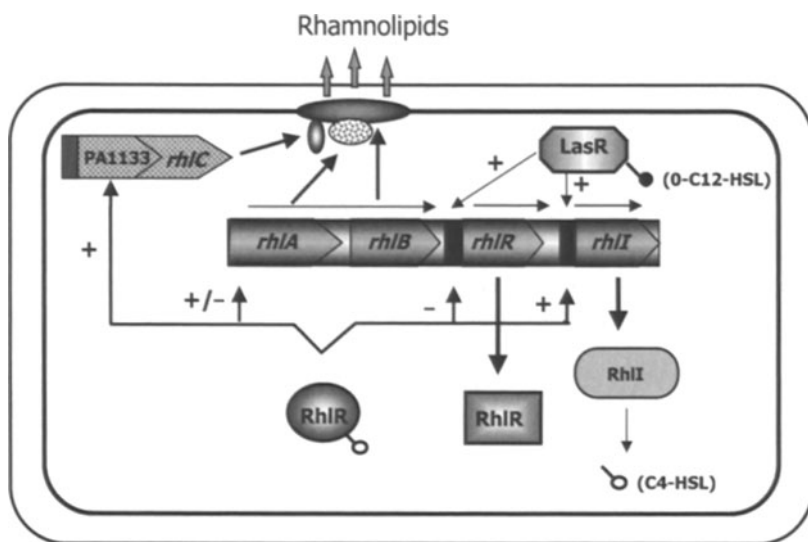


Figure 2. Schematic representation of the structure and regulation of the *rhl* genes encoding RT1 (*rhlAB*) and RT2 (*rhlC*), and the cellular localization of these enzymes. The *rhlR* and *rhlI* genes encode for RhlR transcriptional regulator and RhlI acylhomoserine lactone synthetase RhlI. Autoinducers that interact with RhlR and with LasR, and other transcriptional regulators are shown. The mRNA start site of a gene or operon is located downstream of the promoter that is schematized by the site where the arrows are pointing. The transcription termination site is shown as the head of an arrow in the bar showing the genes in this chromosomal region. The sign + or - refers to the transcriptional regulation effect of RhlR on the promoter located where the arrows are pointing.

regulation through the quorum-sensing response^{30, 31}, as described below. The *rhlC* gene is not linked in the chromosome to other *rhl* genes and forms part of an operon with a gene whose function is not known (Figure 2). The operon containing *rhlC* is regulated at the transcriptional level in a similar manner as *rhlAB* operon³⁸.

3. RHAMNOLIPIDS PRODUCTION IS REGULATED BY THE QUORUM-SENSING RESPONSE

The synthesis of these surfactants is regulated by a very complex genetic regulatory system that also controls different *P. aeruginosa* virulence-associated traits called quorum sensing, which depends on the production of the so-called bacterial autoinducers^{17, 55}.

The most common autoinducers produced by proteobacteria are N-acyl-homoserine lactones (HSL) with side chains of various lengths¹⁷. These small diffusible signaling molecules activate gene expression at high bacterial densities through the interaction with specific transcriptional activators of the LuxR family. In *P. aeruginosa* two autoinducer-mediated transcriptional regulator circuits have been described^{17, 24}. One is dependent on N-3-oxododecanoyl-HSL (3-o-C12-HSL), which is synthesized by the product of the *lasI* gene and promotes the LasR induction of different virulence associated traits including the gene coding for the transcriptional regulator RhlR^{23, 27, 36} and *rhlI*¹³. The second autoinducer-regulated genetic circuit responds to N-butanoyl-HSL (C4-HSL) that is produced by RhlI³¹. This quorum-sensing system promotes, through the activation of RhlR, the expression, among others, of the genes encoding for enzymes involved in rhamnolipid synthesis: the *rhlAB* operon, encoding RT1³⁰ and the *rhlC* gene encoding RT2³⁸. The use of whole genome analysis, including microarrays^{42, 51}, of the quorum-sensing regulated genes in *P. aeruginosa* has shown that hundreds of genes are regulated by this system^{42, 51, 53}. The two *P. aeruginosa* quorum-sensing systems form a very complex regulatory circuit (Figure 3) which has not been completely elucidated.

The transcriptional regulators of the LuxR family bind to a specific DNA sequence in order to activate transcription¹⁷. In the case of *P. aeruginosa* these sequences have been called “las boxes”⁵³. The different DNA motifs which determine that a “las box” is recognized by RhlR or LasR are beginning to be understood^{14, 52}.

We have recently reported that *rhlR* is not only regulated by LasR/3-o-C12-HSL, but that it is subject to a very complex transcriptional regulation²⁷. This gene presents four different promoters (one of which is σ^{54} -dependent), two “las-boxes” and a Vfr (a CRP homolog) binding sequence (Figure 4). RhlR itself seems to repress the expression of one of this

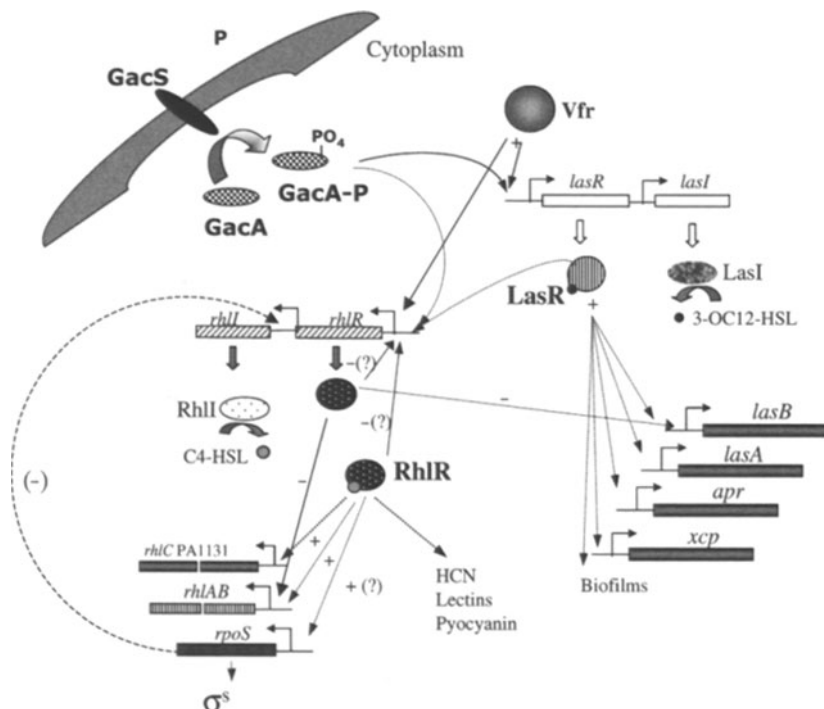


Figure 3. Schematic representation of the *P. aeruginosa* quorum-sensing regulatory network. The symbols in this scheme are the same as in Figure 2. The genes shown in this figure encode the following proteins: *lasA* elastase A; *lasB* elastase B; *apr* alkaline protease; *xcp* proteins belonging to the extracellular protein transport system II.

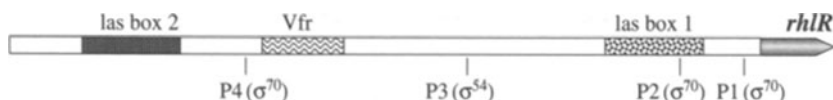


Figure 4. Schematic representation of *rhlR* regulatory region. P1, P2, P3 and P4 depict the four different transcriptional start sites detected. In parenthesis the sigma factor found to be involved in transcription of each promoter is shown. The region marked as Vfr represents the consensus sequence for binding of this transcriptional regulator found in this region. The presence of two putative las boxes is shown.

transcriptional start sites. According to this structure we found that the expression of this gene is tightly regulated by different environmental conditions and that its expression is not only dependent on bacterial density. It is interesting that under some culture conditions *rhlR* expression is only partially dependent on LasR. To our knowledge *rhlR* presents the most complex pattern of transcription regulation described for any of the genes encoding members of the LuxR family of transcription regulators.

We have recently reported²⁹ that RhIR not only activates *rhLAB* promoter when it is bound to C4-HSL, but that it acts as a repressor on this promoter in the absence of its autoinducer (Figure 5). There are other genes in *P. aeruginosa*, like *lasB*, that are fully activated by LasR/3-o-C12-HSL and to a lesser extent by RhIR/C4-HSL³⁵. It has been reported that *lasB* seems to be repressed by RhIR in the absence of C4-HSL². As mentioned above, we have reported evidence suggesting that RhIR represses the expression of its own gene²⁷. To our knowledge, this is the first case of a bacterial transcriptional regulator that activates or represses the same promoter, binding to the same sequence, depending on the binding of a coligand. LasR has been found to bind to *las* boxes and to multimerize only when it is complexed with 3-o-C12-HSL²⁰. According with our finding that RhIR represses the expression of the *rhLAB* promoter in the absence of C4-HSL, it was recently reported that RhIR forms a dimer in the absence and in the presence of this autoinducer⁵⁰.

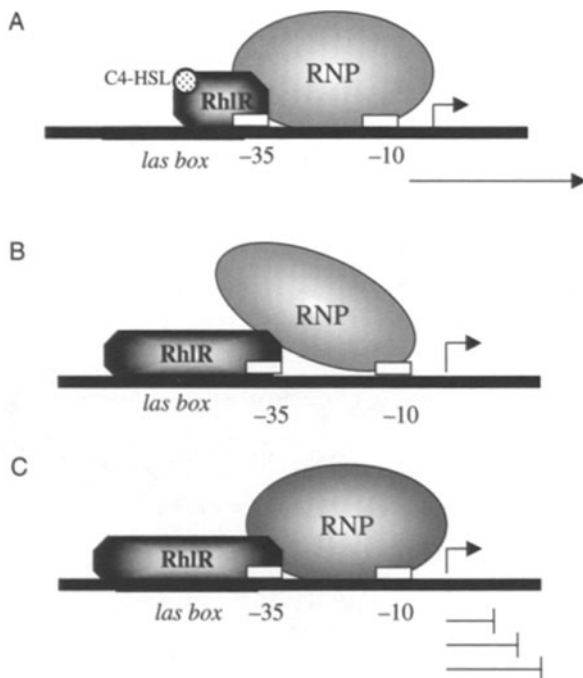


Figure 5. Schematic representation of the transcriptional regulation of the *rhLAB* promoter by RhIR, depending on the ratio of RhIR/RhIR(C4-HSL). When RhIR is bound to its cognate autoinducer C4-HSL it acts a transcriptional activator (A), but when RhIR is not complexed with this autoinducer it acts as a transcriptional repressor, either by blocking transcription initiation (B) or elongation (C). Other elements involved in transcriptional regulation of this promoter are discussed on Section 6.

4. REGULATION OF PRODUCTION OF D-TDP-L-RHAMNOSE AND ITS ROLE IN RHAMNOLIPIDS PRODUCTION

The synthesis of LPS in all gram-negative bacteria uses different activated sugars as precursors. In the case of *P. aeruginosa* (Chapter 1, Volume 3) it has been reported that dTDP-D-glucose, guanosine diphosphate (GDP)-D-rhamnose, GDP-mannose and dTDP-L-rhamnose, among others, are LPS precursors (Figure 6). It has been reported that AlgC plays a central role in the biosynthetic pathway of all these activated sugars⁹. AlgC transforms mannose-6-phosphate to mannose-1-phosphate a precursor of GDP-mannose, and hence of LPS and the exopolysaccharide alginate (Chapter 2, Volume 3), but it also catalyzes the conversion of glucose-6-phosphate to glucose-1-phosphate, a precursor of dTDP-D-glucose and dTDP-L-rhamnose (Figure 6).

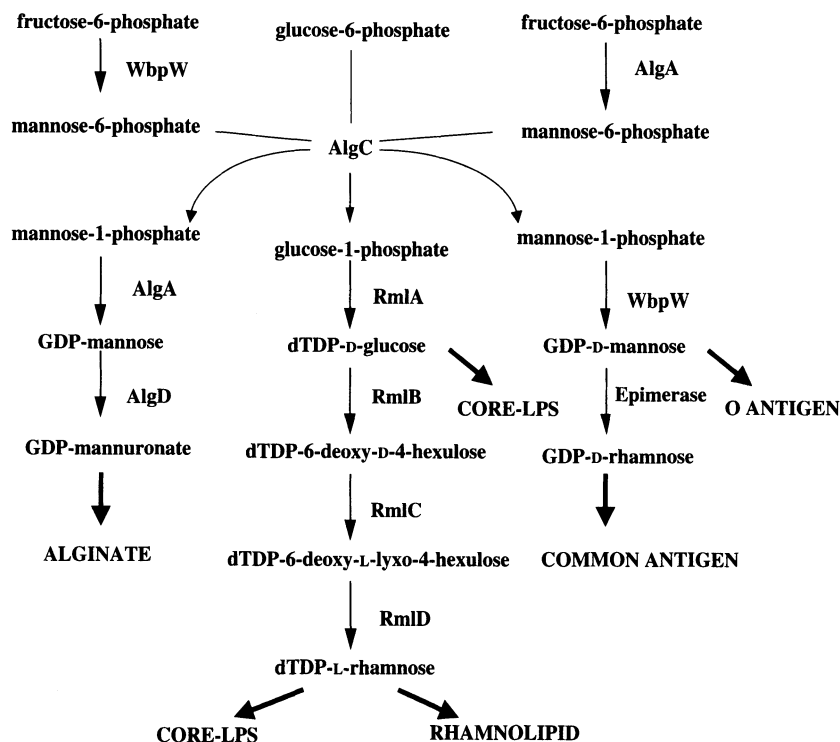


Figure 6. Schematic representation of the dTDP-L-rhamnose, GDP-D-rhamnose, GDP-mannose and GDP-mannuronic acid biosynthetic pathways, and their involvement in the production of alginate, lipopolysaccharide (LPS) and rhamnolipids synthesis. Taken with permission from Olvera *et al.*, *FEMS Microbiol. Lett.*, 179:85–90.

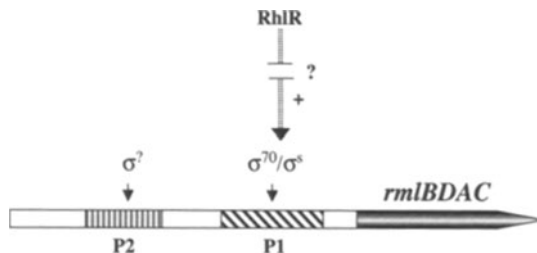


Figure 7. Schematic representation of the *rmlBDAC* transcriptional regulation. The marked areas in the bar represent the two detected promoters P1 and P2. Above these promoters the sigma factors found to be involved in their transcription are shown. A discontinuous arrow is used to point out that RhIR activation is indirect and a question mark is used to show that the elements involved in the direct activation of transcription are unknown.

We described that AlgC through its phospho-gluco-mutase activity is directly involved in rhamnolipids biosynthesis³³.

The dTDP-L-rhamnose biosynthetic pathway has been reported in different bacteria to consist of the conversion of glucose-1-phosphate via dTDP-glucose, dTDP-6-deoxy-D-xylo-4-hexulose and dTDP-6-deoxy-L-lyxo-4-hexulose (Figure 6). In *P. aeruginosa* the enzymes catalyzing these conversions are encoded by *rmlA*, *rmlB*, *rmlC* and *rmlD*, respectively and to be arranged in the operon *rmlBCAD*³⁷. It has been reported that mutations in the *rml* operon on *P. aeruginosa* serotypes that contain L-rhamnose in their LPS, like PAO1, produce truncated LPS molecules³⁷, and are unable to produce rhamnolipids³⁴.

We have recently reported the transcriptional regulation of the *rmlBCAD* operon³⁴. It contains two transcriptional start sites, one of which is only expressed at the stationary phase of growth and seems to be dependent on σ^s for its expression. (Figure 7). The expression of the *rml* operon is dependent on RhIR in an indirect manner, in the absence of RhIR dTDP-L-rhamnose or other metabolic intermediate it seems to accumulate and reduce its expression³⁴.

5. SYNTHESIS OF THE RHAMNOLIPID FATTY ACID MOIETY

Synthesis of the fatty acid moiety of rhamnolipid (Figure 8) separates from the *P. aeruginosa* general fatty acid biosynthetic pathway at the level of the ketoacyl reduction⁷. The enzyme responsible for draining the fatty acid precursors of rhamnolipid from the general biosynthetic pathway is called RhIG and shows significant sequence homology with numerous NADPH-dependent ketoacyl reductases. RhIG is specifically involved in rhamnolipids

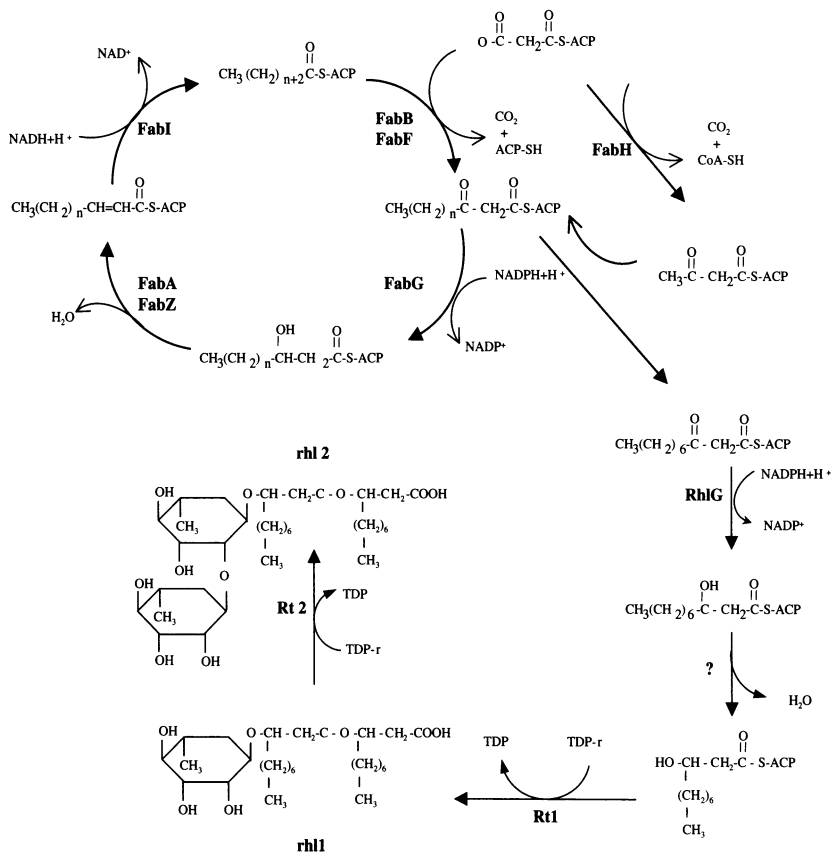


Figure 8. Schematic representation of the fatty acid biosynthetic pathway showing the deduced role of the RhlG protein in the production of rhamnolipids. Initiation of fatty acid biosynthetic cycle, catalyzed by FabH, requires acetyl-CoA and malonyl-ACP to form acetoacetyl-ACP. Subsequent cycles are initiated by condensation of malonyl-ACP with acyl-ACP, catalyzed by FabB and Fab F. In the second step, the resulting β -ketoester is reduced to a β -hydroxyacyl-ACP by FabG. The third step in the cycle is catalyzed by either FabA or FabZ. The fourth and final step is the conversion of trans-2-enoyl-ACP to acyl-ACP, a reaction catalyzed by FabI. Abbreviations used are: TDP-r: thymidine-diphospho-L-rhamnose; Rt1 and Rt2: rhamnosyl transferases 1 and 2, respectively; rhl1: mono-rhamnolipid; rhl2: di-rhamnolipid; β -hdd: β -hydroxydecanoyl-b-hydroxydecanoate.

production and also affects PHA synthesis. General fatty acid content and autoinducer production in a *rhlG* mutant remain unaffected⁷. Accordingly with its role in rhamnolipid production *rhlG* has been shown to contain a “las box” in its regulatory region and thus to be transcriptionally regulated by the quorum-sensing regulators LasR or RhlR⁷.

The fatty acid moiety of rhamnolipids is generally constituted by a dimmer¹⁵, which is the first intermediary in PHA synthesis. However, PHA

synthases can only use fatty-acid-CoA as a substrate³⁹ while RT1 is able to use as substrates free fatty acids^{4, 5} and both ACP- or CoA-linked precursors (Figure 9). There are two types of evidences showing the use of the latter two compounds as substrates. First, *P. aeruginosa* mutants defective in PhaG, the enzyme involved in the transacylation from ACP to CoA, are unable to produce PHA while still able to produce rhamnolipids⁴⁰. The other type of evidence⁶ is the synthesis of mono-rhamnolipid by *Escherichia coli* carrying the *rhlAB* and *rmlBDAC* operons grown on minimal medium with glucose as carbon source (a condition where only ACP-fatty-acids are produced). Similar amounts of mono-rhamnolipid are produced when fatty acids-methyl esters are supplied as carbon source, and the most abundant fatty acid precursor is linked to CoA⁶, suggesting that CoA-fatty acids are also RT1 substrates.

It has recently been published that RhlA catalyzes the synthesis of 3-(3-hydroxyalkanoic acids) that also have surfactant activity and which constitute the lipid moiety of rhamnolipids¹⁵. It thus seem clear that RhlA is involved in the synthesis of the lipid dimmer, while RhlB is involved in rhamnose

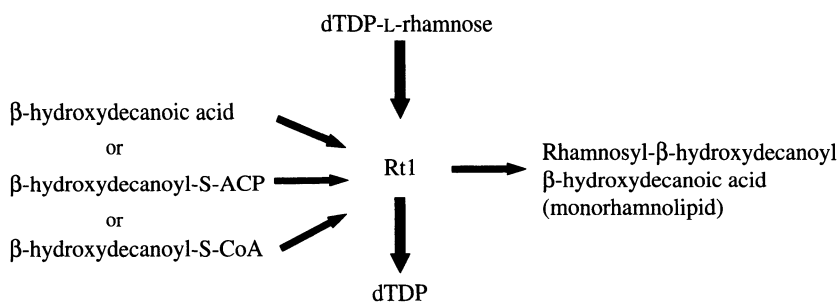


Figure 9. RT1 is able to use different fatty acid derivatives as substrates.

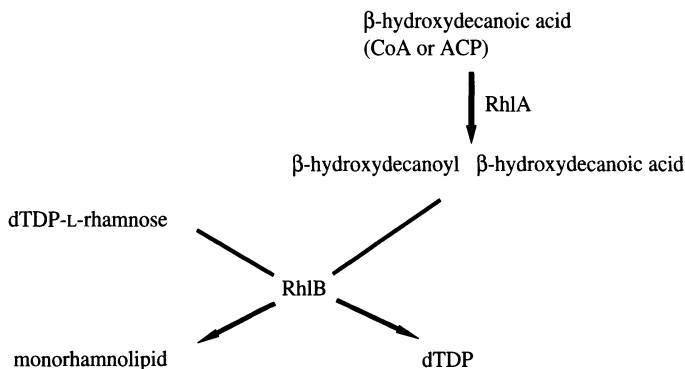


Figure 10. Proposed role of RhlA and RhlB in rhamnolipid and PHA synthesis.

transfer reaction to the lipid acceptor¹⁵ (Figure 10). These results are in accordance with our observation that the lipid moiety of the mono-rhamnolipid produced in *E. coli* expressing the *rhlAB* operon, present the same lipid moiety as those produced in *P. aeruginosa*⁶; and explain the metabolic link between rhamnolipid synthesis and PHA production, since the fatty acids dimmers produced by RhlA seem to be substrates of the enzymes involved in PHA synthesis⁴³.

6. REGULATION OF RHAMNOLIPID PRODUCTION BY NUTRITIONAL FACTORS

Rhamnolipids^{18, 26, 28, 57} as well as other *P. aeruginosa* quorum-sensing regulated traits are only produced on certain culture conditions³ mainly under nutrient limitation. The genetic elements involved in the differential expression of the quorum sensing-dependent traits on nutrient limitation are currently unknown. The dependence of the quorum-sensing response on nutrient availability has been documented for several bacteria^{11, 16, 44, 49}, including *P. aeruginosa*⁵¹. In many bacteria this dependence involves the interaction of the quorum-sensing transcriptional regulators with regulators responding to stress, like σ^s (see ref. [25]). In the case of *P. aeruginosa* there have been several reports which correlate σ^s expression with the quorum-sensing response^{23, 46, 48, 54, 56}, but the precise role of this sigma factor has not been elucidated (Figure 3).

We have reported recently that the *rhlAB* promoter is partially dependent on σ^s for its expression²⁸. In addition we found that this operon is not expressed during the logarithmic phase of growth, even in the presence of RhlR and C4-HSL (Figure 11) when cells are grown on rich media²⁸ or on minimal media supplemented with amino acids, while on minimal media it is expressed since the onset of the culture (Medina and Soberón-Chávez, unpublished results). This behavior is also seen when the expression of the *rhlAB* promoter is studied in the *E. coli* background (Figure 11), showing that a widespread regulatory element in bacteria is responsible for the silencing of the *rhlAB* promoter during the exponential phase of growth²⁷. These results also show that the expression of the *rhlAB* operon is not only dependent on RhlR and C4-HSL, but that nutrient limitation is a necessary condition for its transcription.

To have a more clear picture of the point of regulation of rhamnolipids production in *P. aeruginosa* by nutritional factors, we studied the correlation between *rhlR* expression and rhamnolipids production on different media during the stationary phase of growth (Medina, Sánchez, Aguirre, Juárez, Brom and Soberón, unpublished results). We found that in most conditions the level of *rhlR* expression correlates with the amount of rhamnolipids produced, but that there are exceptions in which the level of C4-HSL seems to be the point of regulation. These results are in agreement with the very complex

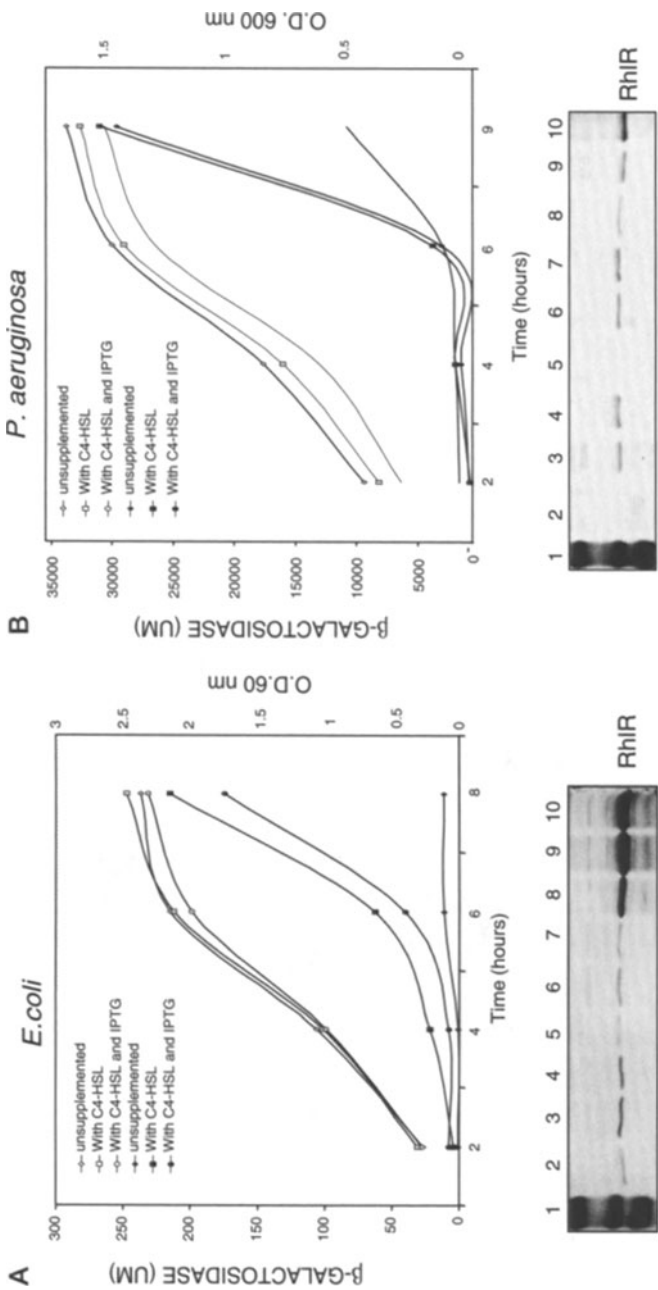


Figure 11. “Exponential silencing” of the *rhlAB* promoter in *E. coli* and *P. aeruginosa*. (A) Expression of an *rhlA::lacZ* fusion along the *E. coli* DH5 α /pECP61.5 growth curve on LB medium, and *P. aeruginosa* PAO1/pCP61.5 growth curve on PPGAS medium, in the presence and absence of 1 mM IPTG and C4-HSL (as stated in the figure). (B) Immunodetection of RhlR on the same conditions shown in (A). Lanes correspond to (1) molecular weight markers, (2), (5) and (8) cultures grown for 2 hr, (3), (6) and (9) cultures grown for 4 hr, (4), (7) and (10) cultures grown for 8 hr. Lanes (2)–(4) show unsupplemented cultures, (5)–(7) show cultures grown with C4-HSL and (8)–(10) show cultures grown with both C4-HSL and IPTG.

structure of *rhlR* promoter²⁷, and with the transcriptional regulation of *rhlI*, which does not seem to be strictly dependent on RhlR¹³.

The differential expression of the quorum-sensing regulon on different environmental conditions⁵¹ and cell growth phases^{42, 51} might explain the existence in *P. aeruginosa* of two quorum-sensing systems.

7. CONCLUSIONS

Pseudomonas aeruginosa is a bacterium that has an environmental lifestyle and at the same time is able to establish pathogenic interactions with a wide variety of hosts^{8, 10, 47}. It is unique since clinical isolates are not genetically distinct from strains isolated from the environment.⁴¹ The understanding of rhamnolipids biosynthesis by *P. aeruginosa* is a model that permits the study of this bacterium from the point of view of its two lifestyles. These biosurfactants are produced along with different virulence associated traits and seem to be also produced in the environment when bacteria are in conditions of nutrient limitation.

We have described the complete *P. aeruginosa* rhamnolipids biosynthetic pathway, showing that it is tightly linked to central metabolic pathways, as are the synthesis of activated sugars and of fatty acids. But also that it shares different enzymatic reactions with the biosynthetic pathways of several bacterial polymers, such as LPS, PHA and alginate.

Accordingly with these characteristics, the regulation of rhamnolipids production at the genetic level is very complex. The quorum-sensing response depending on RhlR and C4-HSL plays a central role, but other regulatory circuits are also involved.

Rhamnolipids have different biotechnological applications, but the complexity of their biosynthetic pathway makes the development of *P. aeruginosa* hyper-producing strains a nonviable approach. There have been some unsuccessful attempts to produce rhamnolipids in heterologous hosts^{30, 32}, presumably due to limitations in RT1 substrate availability. Thus, the development of a metabolic engineering strategy for the production of rhamnolipids in heterologous hosts is an important alternative for the production of these biosurfactants that waits to be developed.

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REFERENCES

1. Al-Tahhan, R.A., Sandrin, T.R., Bodour, A.A., and Maier, R.M., 2000, Cell surface properties of *Pseudomonas aeruginosa*: Effect of rhamnolipid on fatty acid and lipopolysaccharide content. *Appl. Environ. Microbiol.*, 66:3262–3268.
2. Anderson, R.M., Zimprich, C.A., and Rust, L., 1999, A second operator is involved in *Pseudomonas aeruginosa* elastase (*lasB*) activation. *J. Bacteriol.*, 181:6264–6270.
3. Bollinger, N., Hassett, D.J., Iglewski, B.H., Costerton, J.W., and McDermott, T.R., 2001, Gene expression in *Pseudomonas aeruginosa*: Evidence of iron override effects on quorum sensing and biofilm-specific gene regulation. *J. Bacteriol.*, 183:1990–1996.
4. Burger, M.M., Glaser, L., and Burton, R.M., 1963, The enzymatic synthesis of rhamnose containing glycolipids by extracts of *Pseudomonas aeruginosa*. *J. Biol. Chem.*, 238:2595–2602.
5. Burger, M.M., Glaser, L., and Burton, R.M., 1966, Formation of rhamnolipids of *Pseudomonas aeruginosa*. *Meth. Enzymol.*, 8:441–445.
6. Cabrera, N., Olvera, C., and Soberón-Chávez, G., 2003, Mono-rhamnolipid synthesis using *Escherichia coli* as an heterologous host, in preparation.
7. Campos-García, J., Caro, A.D., Nájera, R., Miller-Maier, R.M., Al-Tahhan, R.A., and Soberón-Chávez, G., 1998, The *Pseudomonas aeruginosa* *rhlG* gene encodes a NADPH-dependent b-ketoacyl reductase which is specifically involved in rhamnolipid synthesis. *J. Bacteriol.*, 180:4442–4451.
8. Costerton, J.W., 1980, *Pseudomonas aeruginosa* in nature and disease. In C.D. Sabath (ed.), *Pseudomonas aeruginosa: The Organism, Diseases it Causes and their Treatment*, pp. 15–24. Hans Huber Publishers, Bern, Switzerland.
9. Coyne, M.J. Jr, Russell, K.S., Coyle, C.L., and Goldberg, J.B., 1994, The *Pseudomonas aeruginosa* *algC* gene encodes phosphoglucomutase, required for the synthesis of complete lipopolysaccharide core. *J. Bacteriol.*, 176:3500–3507.
10. D'Argenio, D.A., Gallegher, L.A., Berg, C.A., and Manoil, C., 2002, *Drosophila* as a model host for *Pseudomonas aeruginosa* infection. *J. Bacteriol.*, 183:1466–1471.
11. Dacca-DeLancey, R.R., South, M.M.T., Ding, X., and Rather, P.N., 1999, *Escherichia coli* genes regulated by cell-to-cell signaling. *Proc. Natl. Acad. Sci. USA*, 96:4610–4614.
12. Davey, M.E., Caiazza, N.C., and O'Toole, G.A., 2003, Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1. *J. Bacteriol.*, 185:1027–1036.
13. De Kievit, T., Kakai, Y., Register, K., Pesci, E.C., and Iglewski, B.H., 2002, Role of the *Pseudomonas aeruginosa* *las* and *rhl* quorum-sensing systems in *rhlI* regulation. *FEMS Microbiol. Lett.*, 212:101–106.
14. Deziel, E.F., Lepine, F., Dennie, D., Boismenu, D., Mamer, O.A., and Villemur, R., 1999, Liquid chromatography/mass spectrometry analysis of mixtures of rhamnolipid produced by *Pseudomonas aeruginosa* strain 57RP grown on mannitol or naphthalene. *Biochim. Biophys. Acta-Molec. Cell. Biol. Lipids*, 1440:244–252.
15. Deziel, E.F., Lepine, F., Milot, S., and Villemur, R., 2003, *rhlA* is required for the production of a novel biosurfactant promoting swarming motility in *Pseudomonas aeruginosa* 3(3-hydroxyalkanoic acids (HAAs) the precursors of rhamnolipids. *Microbiol.-UK*, 149:2005–2013.
16. Flavier, A.B., Schell, M.A., and Denny, T.P., 1998, An RpoS (σ^S) homologue regulates acylhomoserine lactone-dependent autoinduction in *Ralstonia solanacearum*. *Mol. Microbiol.*, 28:475–486.

17. Fuqua, W.C., Parsek, M.R., and Greenberg, E.P., 2001, Regulation of gene expression by cell-to-cell communication: Acyl-homoserine lactone quorum-sensing. *Annu. Rev. Genet.*, 35:439–468.
18. Guerra Santos, L., Kappeli, O., and Fiechter, A., 1986, Dependence of *Pseudomonas aeruginosa* continuous culture biosurfactant production on nutritional and environmental factors. *Appl. Microbiol. Biotechnol.*, 24:443–448.
19. Jarvis, F.G. and Johnson, M.J., 1949, A glyco-lipid produced by *Pseudomonas aeruginosa*. *J. Am. Chem. Soc.*, 71:4124–4126.
20. Kiratisin, P., Tucker, K.D., and Passador, L., 2002, LasR, a transcriptional activator of *Pseudomonas aeruginosa* virulence genes, functions as a multimer. *J. Bacteriol.*, 184:4912–4919.
21. Kohler, T., Curty, L.K., Baria, F., Van Delden, C., and Pechère, J.-C., 2000, Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. *J. Bacteriol.*, 182:5990–5996.
22. Lang, S. and Wullbrandt, D., 1999, Rhamnose lipids-biosynthesis, microbial production and application potential. *Appl. Microbiol. Biotechnol.*, 51:22–32.
23. Latifi, A., Foglino, M., Tanaka, K., Williams, P., and Lazdunski, A., 1996, A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhlR (VsmR) to expression of the stationary sigma factor RpoS. *Mol. Microbiol.*, 21:1137–1146.
24. Latifi, A., Winson, M.K., Foglino, M., Bycroft, B.W., Stewart, G.S.A.B., Lazdunski, A., and Williams, P., 1995, Multiple homologues of LuxR and LuxI control the expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1. *Mol. Microbiol.*, 17:333–343.
25. Lazazzera, B.A., 2000, Quorum sensing and starvation signals for entry into stationary phase. *Curr. Opin. Microbiol.*, 3:177–182.
26. Maier, M.R. and Soberón-Chávez, G., 2000, *Pseudomonas aeruginosa* rhamnolipids: Biosynthesis and potential applications. *Appl. Microbiol. Biotechnol.*, 54:625–633.
27. Medina, G., Juárez, K., Díaz, R., and Soberón-Chávez, G., 2003, Transcriptional regulation of *Pseudomonas aeruginosa* *rhlR* encoding a quorum sensing regulatory protein. *Microbiol.-UK*, 149:3073–3081.
28. Medina, G., Juárez, K., and Soberón-Chávez, G., 2003, The *Pseudomonas aeruginosa* *rhlAB* operon is not expressed on the logarithmic phase of growth even in the presence of its activator RhlR and the autoinducer *N*-butanoyl-homoserine lactone. *J. Bacteriol.*, 185:377–380.
29. Medina, G., Juárez, K., Valderrama, V., and Soberón-Chávez, G., 2003, Mechanism of *Pseudomonas aeruginosa* RhlR transcriptional regulation of the *rhlAB* promoter. *J. Bacteriol.*, 185:5976–5983.
30. Ochsner, U.A., Fiechter, A., and Reiser, J., 1994, Isolation, characterization and expression in *Escherichia coli* of the *Pseudomonas aeruginosa* *rhlAB* genes encoding a rhamnosyltransferase involved in rhamnolipid biosurfactant synthesis. *J. Biol. Chem.*, 269:19787–19795.
31. Ochsner, U.A. and Reiser, J., 1995, Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci.*, 92:6424–6428.
32. Ochsner, U.A., Reiser, J., Fiechter, A., and Witholt, B., 1995, Production of *Pseudomonas aeruginosa* rhamnolipid biosurfactants in heterologous hosts. *Appl. Environ. Microbiol.*, 61:3503–3506.
33. Olvera, C., Goldberg, J.B., Sánchez, R., and Soberón-Chávez, G., 1999, *Pseudomonas aeruginosa* algC gene product participates in rhamnolipids biosynthesis. *FEMS Microbiol. Lett.*, 71:85–90.
34. Olvera, C., Treviño-Quintanilla, L.G., Nájera, R., and Soberón-Chávez, G., 2003, The *Pseudomonas aeruginosa* *rmlBDAC* operon, encoding dTDP-L-rhamnose biosynthetic enzymes, is regulated by RhlR in an indirect manner, in preparation.

35. Pearson, J.P., Pesci, E.C., and Iglewski, B.H., 1997, Roles of *Pseudomonas aeruginosa* las and rhl quorum-sensing systems in control of elastase and rhamnolipid biosynthesis. *J. Bacteriol.*, 179:5756–5767.
36. Pesci, E.C., Pearson, J.P., Seed, P.C., and Iglewski, B.H., 1997, Regulation of the las and rhl quorum sensing in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 179:3127–3123.
37. Rahim, R., Burrows, L.L., Monteiro, M.A., Perry, M.B., and Lam, J.S., 2000, Involvement of the *rml* locus in core oligosaccharide and O polysaccharide assembly in *Pseudomonas aeruginosa*. *Microbiology*, 146:2803–2814.
38. Rahim, R., Ochsner, U.A., Olvera, C., Graninger, M., Messner, P., Lam, J.S., and Soberón-Chávez, G., 2001, Cloning and functional characterization of the *Pseudomonas aeruginosa* *rhlC* gene that encodes rhamnosyltransferase 2, an enzyme responsible for di-rhamnolipid biosynthesis. *Mol. Microbiol.*, 40:708–718.
39. Rehm, B.H.A., Kriger, N., and Steibüchel, A., 1998, A new metabolic link between fatty acid de novo synthesis and polyhydroxyalkanoic acid synthesis. *J. Biol. Chem.*, 273: 24044–24051.
40. Rehm, B.H.A., Mitsky, T.A., and Steibüchel, A., 2001, Role of fatty acid de novo biosynthesis in polyhydroxyalkanoic acid (PHA and rhamnolipid synthesis by *Pseudomonads*: Establishment of the transacylase (PhaG)-mediated pathway for PHA biosynthesis in *Escherichia coli*. *Appl. Environ. Microbiol.*, 67:3102–3109.
41. Römling, U., Wingender, J., Müller, H., and Tümmler, B., 1994, A major *Pseudomonas aeruginosa* clone common to patients and aquatic habitats. *Appl. Environ. Microbiol.*, 60:1734–1738.
42. Schuster, M., Lostroh, C.P., Ogi, T., and Greenberg, E.P., 2003, Identification, timing and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: A transcriptome analysis. *J. Bacteriol.*, 185:2066–2079.
43. Soberón-Chávez, G. and Sánchez, R., 2003, The *Pseudomonas aeruginosa* RhlA enzyme is involved in polyhydroxyalkanoate, in preparation.
44. Srinivasan, S., Österling, J., Charlton, T., De Nys, R., Takayama, K., and Kjelleberg, S., 1998, Extracellular signal molecule(s) involved in the carbon starvation response of marine *Vibrio* sp. strain S14. *J. Bacteriol.*, 180:201–209.
45. Stanghellini, M.E. and Miller, R.M., 1997, Biosurfactants: Their identity and potential efficacy in the biological control of zoospore plant pathogens. *Plant Dis.*, 81:4–12.
46. Suh, S., Silo-Suh, L., Woods, D.E., Hassett, D.J., West, S.E.H., and Ohman, D.E., 1999, Effect of the *rpoS* mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 181:3890–3897.
47. Tan, M.-W. and Ausubel, F.M., 2000, *Caenorabditis elegans*: A model genetic host to study *Pseudomonas aeruginosa* pathogenesis. *Curr. Opin. Microbiol.*, 3:29–34.
48. Tanaka, K. and Takahashi, H., 1994, Cloning, analysis and expression of an *rpoS* homologue gene from *Pseudomonas aeruginosa* PAO1. *Gene*, 150:81–85.
49. Thorne, S.H. and Williams, H.D., 1999, Cell density-dependent starvation survival of *Rhizobium leguminosarum* bv. *phaseoli*: Identification of the role of an N-acyl homoserine-lactone in adaptation to stationary-phase survival. *J. Bacteriol.*, 181:981–990.
50. Ventre, I., Ledgham, F., Prima, V., Lazdunski, A., Foglino, M., and Sturgis, J.N., 2003, Dimerization of the quorum sensing regulator RhlR: Development of a method using EGFP fluorescence anisotropy. *Mol. Microbiol.*, 48:187–198.
51. Wagner, E.V., Bushnell, D., Passador, L., Brooks, A.I., and Iglewski, B.H., 2003, Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: Effects of growth phase and environment. *J. Bacteriol.*, 185:2080–2095.
52. Whiteley, M. and Greenberg, E.P., 2001, Promoter specificity elements in *Pseudomonas aeruginosa* quorum-sensing-controlled genes. *J. Bacteriol.*, 183:5529–5534.
53. Whiteley, M., Lee, K.M., and Greenberg, E.P., 1999, Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA*, 96:13904–13909.

54. Whiteley, M., Parsek, M.R., and Greenberg, E.P., 2000, Regulation of quorum sensing by RpoS in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 182:4356–4360.
55. Withers, H., Swift, S., and Williams, P., 2001, Quorum sensing as an integral component of gene regulatory networks in gram-negative bacteria. *Curr. Opin. Microbiol.*, 4:186–193.
56. You, Z., Fukushima, J., Tanaka, K., Kawamoto, S., and Okuda, K., 1998, Induction of entry into stationary phase in *Pseudomonas aeruginosa* by *N*-acylhomoserine lactone. *FEMS Microbiol. Lett.*, 164:99–106.
57. Zhang, Y. and Miller, R.M., 1992, Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* rhamnolipid surfactant (biosurfactant). *Appl. Environ. Microbiol.*, 58: 3276–3282.

ALTERNATIVE RESPIRATORY SUBSTRATES

DENITRIFICATION BY PSEUDOMONADS: CONTROL AND ASSEMBLY PROCESSES

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1. INTRODUCTION

Denitrification is part of the biogeochemical nitrogen cycle driven by prokaryotes. The introduction of reduced nitrogen into the biosphere by nitrogen fixation is reversed by the sequential action of nitrification and denitrification. A complete denitrification process, yielding dinitrogen from nitrate, consists of four respiratory systems utilizing as electron acceptors: nitrate, nitrite, nitric oxide (NO), and nitrous oxide (N₂O) (Figure 1). Backward-running reactions of the N-cycle, nitrate assimilation and nitrate ammonification, as well as a short circuit represented by the anammox process, are not depicted in Figure 1. Nitrate assimilation and ammonification depend on sets of different enzymes as those of nitrate respiration^{75, 92}. The anammox reaction, which reduces nitrite at the expense of ammonia oxidation to form dinitrogen, had been postulated on theoretical grounds. It was recently found in the genus *Planctomyces* and seems to be of ecological importance⁷⁸. Because the process allows the simultaneous removal of oxidized and reduced nitrogen, it attracts considerable attention as a new way of treating wastewater. Although denitrification is a mode of anaerobic respiration, the known

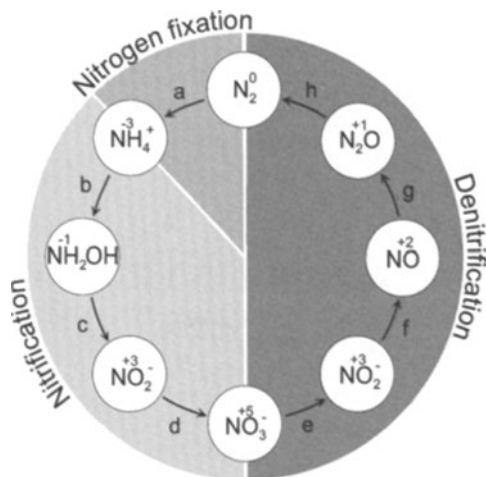


Figure 1. The biogeochemical nitrogen cycle. The scheme shows an idealized pathway of a dinitrogen molecule being recycled by reductive (nitrogen fixation), oxidative (nitrification), and once more reductive reactions (denitrification), and thus undergoing transformations into various inorganic nitrogen species. The formal oxidation state is indicated above each species. Enzymes catalyzing the cycle are (a) dinitrogenases, EC 1.18.6.1 and 1.19.6.1, (b) ammonium monooxygenase, no EC number, (c) hydroxylamine oxidase, EC 1.7.3.4, (d) nitrite oxidoreductase, no EC number, (e) respiratory nitrate reductase, EC 1.7.99.4, (f) respiratory nitrite reductases, EC 1.9.3.2 and 1.7.2.1, (g) nitric oxide reductase, EC 1.7.99.7, and (h) nitrous oxide reductase, EC 1.7.99.6.

denitrifying prokaryotes are nearly exclusively aerobic organisms which express the process facultatively. A main theme of denitrification research, therefore, is how bacteria perceive the environmental signals to turn on the alternative gene programs and regulate the coordinate expression of the denitrification apparatus.

The substantial advances made in the understanding of the denitrification system during the past two decades have been summarized in several reviews. Two treatises cover the entire process with emphasis on the biochemistry and bioenergetics¹⁴, or the cell biology and molecular genetics projected against the diversity of denitrifying organisms¹⁵⁹. Physiological genetics of dissimilatory nitrate reduction by bacteria have been compared with the assimilatory pathway⁹². Formation of NO from nitrite by the two biochemical variants of nitrite reductases²⁹ as well as NO reduction¹⁴² were discussed from structural and mechanistic viewpoints. The known structures of denitrification enzymes have been reviewed⁹³. X-ray structures of respiratory nitrate reductase and NO reductase are still missing. For the latter enzyme a feasible model exists at least, based on the similarity with cytochrome-*c* oxidase¹⁵⁹.

A modeling approach is possible because of the structural and evolutionary relationships between the two enzymes^{115, 133, 158}.

2. DENITRIFICATION BY PSEUDOMONADS

The genetic inventory of pseudomonads with respect to the nitrogen cycle consists in the enzymes *e* through *h* shown in Figure 1. Many species are capable of anaerobic nitrate denitrification and usually they also have the capability to assimilate nitrate. It is likely that Gayon and Dupetit, the codiscoverers of denitrification, already studied the process with a pseudomonad¹⁵⁹. Pseudomonads contribute a large assemblage to the denitrifying prokaryotes^{157, 159}. They are amenable to genetic manipulation and, since the process cannot be investigated with enterobacteria, seem to be the logical choice for the study of denitrification. The most commonly isolated denitrifiers from temperate soils are fluorescent pseudomonads which have the ability of complete nitrate denitrification to dinitrogen⁴⁶. This is seen as their selective advantage and might explain in part the observed prevalence³⁵. The biovars B, C, and F of *Pseudomonas fluorescens* harbor denitrifying bacteria. The strain of *P. fluorescens* PfO-1 being sequenced currently is of importance as a plant pathogen, but is nondenitrifying.

Pseudomonas stutzeri and *Pseudomonas aeruginosa* have been used prominently to explore the denitrification process and central findings were made first investigating a pseudomonad. *P. stutzeri* (initially named *Bacillus denitrificans* II by its discoverers R. Burri and A. Stutzer in 1895) is a vigorous denitrifier and grows on nitrate, nitrite, and nitrous oxide (N₂O). *P. stutzeri* was the source organism for the discovery of the enzymes and genes for N₂O reduction and NO reduction. The ZoBell strain (formerly *Pseudomonas perfectomarina*) is a marine isolate off the California coast, although *P. stutzeri* seems not be a prevalent inhabitant of the marine environment. Quantitation of a denitrification specific marker reveals a greater abundance of *P. stutzeri* in lake sediments and ground water⁵¹. A new denitrifying species, *Pseudomonas balearica*, was isolated from the Mediterranean Sea¹³.

Specific aspects of denitrification have been studied with several other pseudomonads. Of the denitrifying *P. fluorescens* C7R12 the *nos* gene cluster has been sequenced⁹⁹. *Pseudomonas aureofaciens* and a taxonomically yet to be defined *Pseudomonas* sp. G-179 have been shown to harbor a copper-containing nitrite reductase (Cu-Nir)^{11, 164}. *Pseudomonas nautica* strain 617 provided the source of N₂O reductase (N₂OR) for crystallization and structure determination¹⁸. Note, however, that the type strain of *P. nautica* is identical with that of *Marinobacter hydrocarbonoclasticus*¹²⁵. *Pseudomonas putida* has been used as the nondenitrifying host for the expression of denitrification genes from *P. aeruginosa*¹¹⁹ and *P. stutzeri*¹⁴⁸.

3. DENITRIFICATION GENES, IDENTIFICATION, AND ORGANIZATION

It is clear now that denitrification genes are organized in several clusters on the chromosome. Three loci have been identified in *P. stutzeri* and four in *P. aeruginosa* (Figure 2). The genes for assimilatory (*nas*) and dissimilatory (*nar* and *nap*) nitrate reduction are unlinked in separate loci, whereas the *nir* genes for nitrite respiration and the *nor* genes for NO respiration are vicinal. This holds also for other denitrifiers. The denitrification gene clusters harbor, in addition to the structural information for the enzymes, functions for metal

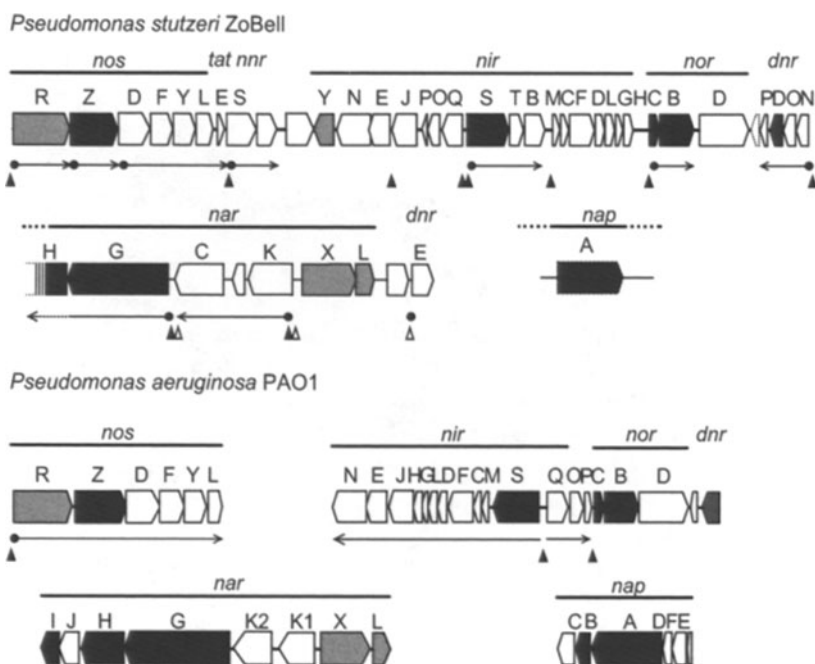


Figure 2. Denitrification gene loci of *P. stutzeri* and *P. aeruginosa*. Arrow boxes denote the relative size of open reading frames and the direction of transcription. Homologous genes of the two species carry the same designation. Solid arrow boxes indicate reductase structural genes; regulatory genes are identified by gray boxes; white boxes denote ancillary functions explained in the text. The orientation of gene clusters of *P. aeruginosa* corresponds to that of the genome with the *nos* genes encoded by the + strand. Mapped transcription start sites are indicated by dots, putative sites of Fnr boxes by solid triangles, NarL boxes by open triangles. The extent of transcripts is shown by arrows. The broken outline for *napA* of *P. stutzeri* indicates lack of sequence information.

processing, cofactor synthesis, electron donation, protein maturation, assembly processes, transport, and regulation. As a matter of fact, indispensable ancillary functions comprise the majority of denitrification genes (Figure 2). Currently about 40 genes have been identified each of *P. stutzeri* and *P. aeruginosa*. The individually sequenced DNA fragments and the recent expansion of information from several genomes of denitrifying bacteria allow a comparative study of gene organization⁹⁷. The arrangement of denitrification genes and their relative location within individual clusters is considerably conserved. Overall they follow the pattern that prokaryotic genes involved in the same metabolic pathway are clustered. It is also gratifying to see that the individual analyses and mapping carried out prior to the genomic era are confirmed by the complete genome sequence of *P. aeruginosa*¹²⁶.

The isolation of the genes for N₂O utilization (*nos*) marked the beginning of unraveling the genetic background of denitrification. The gene for N₂OR, *nosZ*, was the very first gene of known structure in the field of anaerobic N-oxide metabolism including nitrate respiration¹³⁵. It was mapped by transposon Tn5 insertions and identified by expression in *Escherichia coli*. The *nirS* gene for cytochrome-*cd*₁ nitrite reductase was isolated from *P. aeruginosa* using oligonucleotide probes designed from the amino acid sequence of the purified enzyme¹²⁰. The same approach was used to identify the *norCB* genes encoding the cytochrome-*bc* NO reductase complex of *P. stutzeri*¹⁶. The structural gene for Cu-Nir, *nirK*, was identified in *Pseudomonas* sp. G-179 by tagging the wild-type gene with Tn5¹⁵¹ or in *P. aureofaciens* by screening an expression library with an anti-NirK antiserum⁴⁸. The structural genes encoding the two subunits NapA and NapB of the periplasmic nitrate reductase were identified in *Ralstonia eutropha*¹¹⁸. Homologues of *napA* have been detected by DNA-DNA hybridization in *P. aeruginosa* and *P. stutzeri*¹³⁸. Compared with the genetic information available for denitrification *sensu stricto*, our knowledge about the nitrate-respiring system of the pseudomonads is still limited, which extends also to comparative sequence information and gene organization of *nas* and *nap* gene clusters.

The majority of the 35 genes in the principal denitrification cluster of *P. stutzeri* are transcribed in the same direction. They form probably 12 transcriptional units, of which little over half have been identified experimentally. Polycistronic transcripts exist for *norCB*¹⁶¹, *nirSTB*⁵³, *dnrNODP*¹³⁹, *nosDFYLtatE*, and the *nnrS* operon⁶⁰. The transcriptional organization in *P. aeruginosa* is less complex than that of *P. stutzeri* (Figure 2). Operon structures deduced for the former organism rest on indirect evidence by demonstrating lack of internal promoter activity of gene constructs with *lacZ* or *xyleE* fusions. The *nos* genes form a single *nosRZDFYL* operon⁷. The *nir* genes may be transcribed as a large operon comprising 11 genes, *nirS* through *nirN*⁶⁹, and the tricistronic operon, *nirQOP*³. As far as the multicistronic operons of

denitrification genes encode disparate functional roles, they prompt the question by which means the required balanced concentrations of gene products for proper cellular functioning are adjusted.

Transcript start sites have been mapped for the *P. stutzeri* genes *nosR*, *nosZ*, and *nosD*³¹, *nnrS*⁶⁰, *norCB*¹⁶¹, *dnrD*¹³⁷, *nirS*⁵³, and *narG* and *narK*⁵². This allows the localization of recognitions motifs for transcriptional regulators (Figure 2). For *P. aeruginosa* denitrification genes, only the transcript start site of *nosR* is known so far⁷. Several different types of regulators are encoded within the *nar*, *nir*–*nor*, and *nos* clusters by the genes *narXL*, *dnr*, *dnrD*, *nirY*, and *nosR* (Figure 2). *narXL* encodes a nitrate sensitive two-component sensor-regulator system. The *dnr* and *dnrD* orthologues of *P. aeruginosa* and *P. stutzeri*, respectively, encode NO-responsive regulators which are considered to function as the master switch for the expression of the denitrification system¹⁶⁰. Both are found downstream and in the opposite direction of the *nor* operons of *P. stutzeri* and *P. aeruginosa*. The term Dnr describes now a subgroup of homologous regulators within the Crp-Fnr superfamily; thus, renaming the *P. aeruginosa* gene would help nomenclatorial transparency. *nosR* encodes a regulator for *nosZ* expression. *nirY* encodes a LysR-type regulator whose target genes need to be identified. Regulatory genes for denitrification reside also outside the known denitrification loci. *anr* of *P. aeruginosa* encodes a global transcriptional activator for anaerobic metabolism including denitrification⁴⁵; its homologue in *P. stutzeri* is *fnrA*. The genes comprising the Anr and FnrA regulons in these two bacteria are not identical¹³⁴.

Denitrification genes also form part of the quorum-sensing regulon. The autoinducer *N*-(3-oxododecanoyl) homoserine activates *nirS*, *norBD*, and *napCB* of *P. aeruginosa*, but represses *nirMCFLJN* and *nosRZDFLYL*¹⁴⁰. In physiological terms this could mean that nitrite denitrification terminates under certain conditions at the level of N₂O. The apparent opposite regulatory behavior of the *nirS* gene and genes for heme-*d*₁ biosynthesis is puzzling, considering the presumed operon structure of the *nir* gene set⁶⁹ and the requirement of either gene functions for nitrite respiration.

4. NITRATE RESPIRATION AND MEMBRANE TOPOLOGY OF DENITRIFICATION

Complete denitrification is an assembly of four respiratory systems, showing different degrees of autonomy. Nitrate respiration and N₂O respiration may function as independent processes, each satisfying the entire energy need of a bacterium. Individually controlled gene clusters for *nar* and *nos* support this notion. In contrast, the reduction of nitrite and the reduction of NO

are coupled by a common regulator of the Crp-Fnr family, such that the expression of nitrite respiration is concomitant with that of NO respiration to avoid accumulation of NO.

Upon induction, the oxidoreductases of denitrification are being added to the aerobic respiratory chain whose central components remain functional. The aerobic respiratory chain of *P. aeruginosa* comprises primary dehydrogenases, coenzyme Q-9, the cytochrome-*bc*₁ complex, cytochrome *c*₅₅₁, and several terminal oxidases¹⁵⁵. The genome reveals clearly four types of oxidases: cytochrome *aa*₃ (*cox*), cytochrome-*o* ubiquinol oxidase (*cyo*), cytochrome *cbb*₃ (*cco*), and a cyanide insensitive oxidase (*cio*)¹²⁶. The *cco* operon is duplicated in two adjacent *ccoNOQP* sets¹⁰⁰, and there are several more genes whose products show similarity to oxidases and need further analysis. Several oxidases have been purified^{44, 84, 109}. The global anaerobic regulator Anr has been shown to affect the aerobic inventory of oxidases and is proposed to act as a repressor for aerobic respiration¹¹⁰.

The basic composition of the respiratory chain of *P. stutzeri* is assumed to be similar to that of *P. aeruginosa*, although there is no evidence for cytochrome *aa*₃ but for cytochrome *cbb*₃^{137, 138} and a cytochrome-*co* oxidase⁵⁸. Other than in *P. aeruginosa* the *ccoN* operon is not duplicated. The *cbb*₃-type oxidase has been isolated. It consists of a 114.1 kDa CcoNOP complex of three subunits in equimolar stoichiometry (53.2, 23.5, and 34.9 kDa). CcoQ is not part of the purified protein^{100, 132}. The oxidase is expressed preferentially under O₂-limited conditions and is probably a high-affinity oxidase not operative in oxygen-saturated cells¹³⁷. Figure 3 shows the composition and topology of the respiratory chain of *P. stutzeri* or *P. aeruginosa*.

A dual signaling pathway mediating nitrate (in part also nitrite) and oxygen responses is operative in pseudomonads for the expression of nitrate respiration. Activating the *narGHJI* operon involves regulators of the NarXL two-component and the Crp-Fnr families. Nitrate added to O₂-limited cells first induces respiratory nitrate reductase which generates nitrite⁷³. This, in turn, is thought to trigger the NO-specific signal transduction pathway by forming NO enzymatically or nonenzymatically. The genes for the nitrate sensor, NarX, and the response regulator, NarL, are located upstream of the *nar* operon in *P. stutzeri*⁵², *P. aeruginosa*¹²⁶, and *P. fluorescens*⁹⁹. Knockout mutagenesis of *narXL* prevents *nar* expression in *P. stutzeri*, without affecting the other oxidoreductases of denitrification. The *Pseudomonas* NarXL regulatory system is assumed to be structurally and functionally similar to that of *E. coli*³³. Although it has not yet been shown experimentally here, the 7-2-7 recognition sequences, with the heptameric TACYXMT (Y = C or T; M = A or C) consensus half site, are the likely sites of binding of phosphorylated NarL for gene activation⁵². They are centered at -100 and -94.5 of the *narG* and *narK* promoters, respectively (Figure 4). Note that NarL sites show

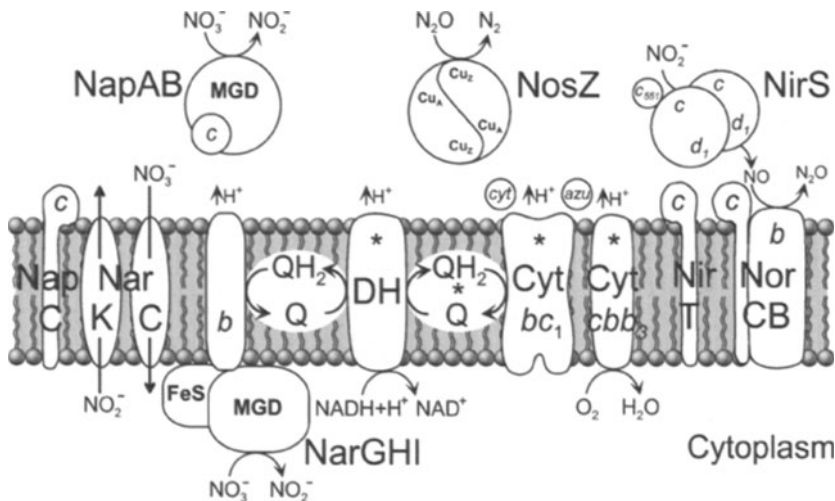


Figure 3. Components and membrane topology of the anaerobic electron transfer chain of denitrification. The situation is depicted for O_2 -limiting conditions of *P. stutzeri*, which leads to the expression of the denitrification system in the presence of nitrate. Components of the aerobic respiratory chain consisting of NADH dehydrogenase (DH), the Q-cycle (QH_2/Q), cytochrome bc_1 (Cyt bc_1), and a terminal oxidase (Cyt cbb_3) are marked with asterisks. No compositions of these complexes are being detailed. The denitrification system comprises respiratory nitrate reductase (NarGHI), nitrite reductase (NirS), NO reductase (NorCB), and N_2O reductase (NosZ). NapAB is the periplasmic nitrate reductase which has a membrane-bound electron-donating component, NapC. Proton translocation ($\uparrow H^+$) is driven by a redox loop mechanism (nitrate reduction) or by the Q-cycle and proton pumping complexes. Abbreviations: FeS, iron-sulfur centers; b , c , and d_1 , heme b , heme c , and heme d_1 , respectively; MGD, molybdopterin guanine dinucleotide; *cyt* and *azu*, unspecified periplasmic c -type cytochrome and blue Cu protein, respectively, both accepting electrons from the cytochrome- bc_1 complex; c_{551} , cytochrome c_{551} . Except for NirT (which is absent) the scheme is valid also for *P. aeruginosa*. NirT and NapC are homologues of a tetraheme protein family involved in electron donation from quinol to the periplasm¹¹⁴. Putative nitrite or nitrate transporters are termed NarK and NarC, respectively, which correspond in *P. aeruginosa* to NarK₁ and NarK₂; their functional assignment follows reference [91].

considerable variability with respect to location and orientation of half sites toward the start of transcription of target genes³³.

Expression of the *nar* operon depends also on a signal generated by low oxygen tension or anoxia. This response is mediated by the Fnr-type transcription factor Anr in *P. aeruginosa*¹⁵². The homologous anaerobic regulator FnrA of *P. stutzeri* apparently does not act on the *nar* operon but on a number of other anaerobically expressed genes, among them the *ccoN* operon for cytochrome *cbb₃* and the arginine deiminase pathway¹³⁷. It is not uncommon that a bacterium harbors multiple members of Crp-Fnr regulators⁷² and not all Fnr-like proteins of *P. stutzeri* may have been identified yet. Anr and

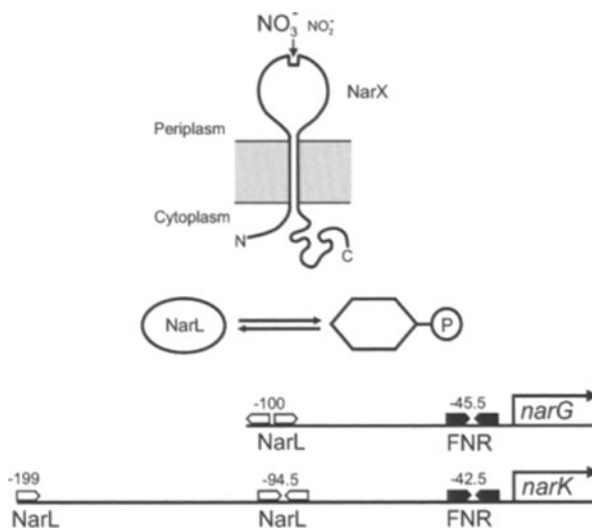


Figure 4. The NarXL two-component system for nitrate sensing. *nar* gene activation requires the NarX transmembrane-acting sensor with a periplasmic domain for interaction with the signal molecule. The sensor has also a minor specificity for nitrite. The cytoplasmic kinase domain of NarX acts on the NarL regulator, which in its phosphorylated form binds to heptameric recognition sites of target genes. A signal from low oxygen, which is mediated by an Fnr-type regulator, is assumed to act concertedly with nitrate regulation. In *P. aeruginosa* this is Anr¹⁵². The positions of the regulatory motifs (NarL, Fnr) are given for *P. stutzeri* as the distances of the center of the motifs from the transcription start sites. Note the inverted orientations of NarL half sites in the two target promoters⁵².

FnrA belong to the superfamily of Crp-Fnr transcription regulators. From their primary structure one can predict that both are [4Fe-4S] proteins which regulate target genes by an oxygen-triggered disassembly-assembly mechanism as established for Fnr of *E. coli*⁷⁰.

The *narK* genes and their homologues encode transport proteins. The fact that there are two genes in *P. aeruginosa* and *P. stutzeri* (*narK*₁/*narK*₂ and *narK*/*narC*, respectively) is interpreted to satisfy the need for nitrate uptake and nitrite efflux. Respiratory reduction of nitrate occurs at the inner side of the cytoplasmic membrane necessitating movement of nitrate and nitrite across the membrane in opposite directions. The transport of these oxyanions in prokaryotes has been reviewed⁹¹.

Respiratory nitrate reductases have been purified from *P. aeruginosa*^{20, 49}, *P. stutzeri*^{15, 59}, *P. fluorescens*⁹⁸, and "*Pseudomonas denitrificans*"⁶⁵. Overall the enzyme properties are highly similar to those of the well-studied nitrate reductase from *E. coli*, which is also apparent in the considerable similarity in primary structure. *Pseudomonads* can also harbor a dissimilatory nitrate

reductase, which is encoded by the *nap* system and resides in the periplasm^{126, 138}. Remarkably, in *Pseudomonas* sp. G-179 it is this enzyme which initiates denitrification¹¹. Thus, a *nap* mutant is unable to grow with nitrate under anaerobic conditions. A similar case for such a Nap function in denitrification (which, however, is not a generalized one) was found in strain IL106 of *Rhodobacter sphaeroides*⁸³. The biochemistry and physiology of the Nap nitrate reductase system has been reviewed elsewhere¹⁰³.

5. NITRIC OXIDE HOMEOSTASIS, FORMATION AND REDUCTION OF A TOXIC METABOLITE

NO in denitrifying bacteria is a reaction product and the substrate of respiration at the same time. This is established from the identification and purification of the NO reductase complex, mutational inactivation of the *norCB* genes, demonstration of the buildup of a steady state concentration of NO during denitrification, and proof for the occurrence of NO in the environment¹⁵⁹. The steady state concentrations of free NO during denitrification is in the low nanomolar range; for example, nitrate-denitrifying *P. aeruginosa* develops a concentration of 1–2 nM NO⁶⁷. The bacterial cell must keep NO within narrow limits of an obligatory cell metabolite and an undesirable toxic compound with reactivity toward multiple cellular systems. NO at 0.5 mM completely prevents the transcription of the operons for nitrite reductase (*nirSTB*) and NO reductase (*norCB*) in *P. stutzeri*¹³⁹ and even in denitrifiers, which are adapted to NO, the loss of NO reductase is a lethal event due to NO accumulation^{16, 28}.

NO homeostasis is determined in denitrifying bacteria by NO production from nitrite and sink reactions involving NO reductase^{58, 161}, cytochrome-*cbb*₃ oxidase⁴², and flavohemoglobin¹⁰². A novel flavorubredoxin-type NO reductase may contribute to NO homeostasis and NO detoxification in many bacteria⁴⁷, except the pseudomonads. The genomes of *P. aeruginosa*, *P. fluorescens*, *P. putida*, and *P. syringae* do not carry orthologues of the *norV* gene encoding this type of reductase.

NO reductase of the pseudomonads is a cytochrome-*bc* type enzyme. The cytochrome *c* subunit (NorC), whose heme-binding domain is periplasmic, is thought to provide electrons from a periplasmic donor molecule to the catalytic cytochrome *b* subunit (NorB). The NO reductase genes are organized as a *norCB* operon. *nirQ* and *norD* are also required for an in vivo functional NO respiration but their roles are unknown¹⁵⁸. The most remarkable feature of NO reductase of cytochrome *cb*-type is its structural similarity with the catalytic subunit of heme-copper oxidases. Oxygen activation by this enzyme proceeds at a heme-Cu binuclear site, whereas NO reductase catalysis is based

on a heme-Fe binuclear site²³. No experimental evidence exists that NO reductase would have proton-pumping properties.

The enzymatic NO generators of denitrifiers are two types of respiratory nitrite reductases. They are found on an either/or basis in denitrifying bacteria without recognizable taxonomic pattern of dissemination. The *nirS* gene encodes the tetraheme cytochrome-*cd*₁ nitrite reductase in *P. aeruginosa* and *P. stutzeri*. Cu-Nir has been found in *P. aureofaciens* and *Pseudomonas* sp. G-179 as well as many other denitrifiers⁹⁷. The remarkable evolutionary dichotomy with respect to these two metalloenzymes extends also to the archaea. *Pyrobaculum aerophilum* harbors *nirS*⁴¹ whereas Cu proteins have been isolated from halobacteria^{63, 64}. Crystal structures of both types of nitrite reductases have been determined (reviewed in ref. [93]). A study of nitrite reductase distribution, based on immunological cross-reactivity, has indicated that the Cu enzyme exists in more diverse environments than cytochrome *cd*₁²⁷. Efforts to determine the distribution of both reductases in natural habitats have advanced to specific DNA probes, real-time PCR, and *in situ* fluorescence hybridization. A verdict is not out yet whether a distributional pattern can be rationalized with a selective advantage on a biochemical basis. However, these studies indicate that nitrite reductases are more diverse in nature than what is reflected in the frequently used laboratory strains^{105, 112}. Cu-Nir has been isolated from *P. aureofaciens* as an enzyme species largely depleted of type 2 Cu¹⁶⁴, but since Cu-Nir has not been studied further in pseudomonads the reader is referred to reviews covering this type of reductase in other denitrifying bacteria^{29, 93, 142, 159}.

Inactivation of *nirS* results simultaneously in decreased NO reduction and a low level expression of NO reductase in *P. aeruginosa*, *P. stutzeri*, and *P. fluorescens*^{6, 96, 150}. Complementation of a cytochrome-*cd*₁ deficient mutant with *nirK* led to the finding that NO is a signal molecule for the induction of the denitrification apparatus^{96, 161}. Direct activation of gene expression by NO was shown for the *nirK* promoter of *R. sphaeroides*^{79, 129} and subsequently established for a number of other NO-responsive promoters. NO is now viewed as the central signal molecule for the expression of the denitrification apparatus¹⁶⁰. The effective concentration of free NO as an inducer for the transcription of the *nirSTB* and *norCB* operons of *P. stutzeri* is in the range of 5–50 nM NO¹³⁹. This NO concentration is close to that building up during steady state nitrate denitrification.

5.1. Cytochrome-*cd*₁ Nitrite Reductase, the Best Studied Denitrification Enzyme

Most of the work carried out on cytochrome *cd*₁ made use of the enzyme from *P. aeruginosa*, which is the best studied denitrification enzyme.

Cytochrome *cd*₁ was the first oxidoreductase of denitrification to be discovered. Biochemical and physiological studies established its function as a nitrite reductase for the denitrification process¹⁴⁹. The enzyme is structurally simple compared to cytochrome-*c* oxidase and may serve as a model for oxygen activation. Both cytochrome *cd*₁ and Cu-Nir exhibit some oxygen reductase activity.

The crystal structure of cytochrome *cd*₁ was obtained first with the *Paracoccus pantotrophus* protein⁴³. Because of taxonomic uncertainty the source organism was initially referred to in the literature as *Paracoccus denitrificans* GB17 or *Thiosphaera pantotropha*. The structure of the *P. aeruginosa* enzyme was solved by molecular replacement techniques⁹⁴. Cytochrome *cd*₁ is functionally a dimer where each monomer of about 60 kDa has two domains. The heme-*d*₁ domain consists in a striking β -propeller structure with eight blades. We will find this structural motif with seven blades again in N₂OR. Viewed end-on, heme *d*₁ is located at the center of the propeller-like structure. The heme-*c* domain consists in a predominantly α -helical arrangement. Heme *d*₁ binds nitrite and receives electrons from the heme *c*-binding domain. Nitrite reductase of *P. aeruginosa* (but not of *P. pantotrophus*) exhibits domain exchange, that is, the N-terminal "arm" of each heme-*c* domain of subunit 1 reaches over to its counterpart heme-*d*₁ domain of subunit 2⁹⁴.

The oxidized *P. pantotrophus* structure is that of a resting enzyme. Reduction of this enzyme changes the His–His ligation of heme *c* to His–Met and releases Tyr25 from heme *d*₁¹⁴³. The Tyr25-region and associated ligand movement seems to be a peculiar case of *P. pantotrophus*. The equivalent region is deleted in two strains of *P. stutzeri*^{66, 123}. Mutation of Tyr10 to phenylalanine of the *P. aeruginosa* cytochrome *cd*₁, which is topologically homologous to Tyr25 of *Paracoccus*, does not affect the enzymatic and spectroscopic properties of the enzyme, excluding this residue from the catalytic pathway³⁰. Recently it has been shown that Tyr25 is not required by the *P. pantotrophus* enzyme for catalysis. An engineered Tyr25Ser mutant is catalytically competent and does not require a reductive activation step prior to the reaction cycle⁵⁰. The movement of the tyrosine residue must have therefore a rationale other than the initially proposed participation in catalysis.

Cytochrome *cd*₁ is an excellent system to study the kinetics of intramolecular electron transfer between the two heme groups. Intramolecular electron transfer is controlled by an allosteric effect involving the two subunits and depending on the reduction state of the enzyme⁴⁰. Electron donors for cytochrome *cd*₁ are small cytochromes such as cytochrome *c*₅₅₁ (the *nirM* product) or the *nirC* product⁵⁵. These donors take up electrons from the cytochrome-*bc*₁ complex. A cytochrome-*bc*₁ mutant loses its ability to respire nitrite although catalytically active nitrite reductase is synthesized⁵⁶. Azurin also is a candidate for an electron donor but physiological evidence does not

strongly support a role in nitrite denitrification. Rather, this low mass Cu protein seems involved in stress response and in stationary phase survival¹³⁶, though a dual role may also be the case. Recognition among electron transfer partners is suggested to proceed via a hydrophobic-patch interaction¹⁴⁴. Not being highly discriminatory, this type of recognition provides a rationale for the interchangeability of several electron donors.

5.2. Reductase Assembly and Heme- d_1 Synthesis

The electronic spectrum of cytochrome cd_1 is characterized by the long wavelength absorption band around 650 nm of heme d_1 and a weak Soret absorption maximum around 460 nm in addition to the heme- c absorption characteristics. The heme- d_1 macrocycle is a 3,8 dioxo-17-acrylate-porphyrindione with a set of oxo, methyl, and acrylate substituents making it characteristic for denitrification and unique among the tetrapyrroles (Figure 5). The structure has been proven from chemical synthesis¹⁴⁷. The distinct redox properties of heme d_1 are assumed to be due to the oxo groups in conjugation with the macrocycle.

The assembly of cytochrome cd_1 is a process of general biological significance. It consists in the translocation of the protein across the membrane, the concomitant or separate transport of the prosthetic groups into the periplasm, covalent binding of heme c , insertion of the noncovalently bound heme d_1 , and folding of the protein into its mature form. A functional cytochrome cd_1 , carrying both types of heme groups, cannot be heterologously expressed from *nirS* alone. Expression of *nirS* in *P. putida*¹¹⁹, *E. coli*, or *P. aureofaciens*⁴⁸ yields in each instance semi-apocytochrome cd_1 with only heme c attached to the protein. Functions required for heme- d_1 synthesis apparently are not provided by the heterologous hosts. Denitrification is dependent on increased cytochrome synthesis, requiring besides cytochrome cd_1 also several c -type cytochromes. A cytochrome c -deficient mutant of *P. aeruginosa*¹⁰⁹ loses its capability to grow anaerobically on nitrate. The biogenesis of c -type cytochromes involves a multifunctional heme transport and heme lyase complex¹²⁸ and it seems necessary that these cytochrome- c maturation functions act also on the cytochrome- c moiety of cytochrome cd_1 . Synthesis of holo-cytochrome cd_1 needs in *P. stutzeri* both the Sec protein transport system (for reductase export) and the Tat twin arginine system (for the NirD component of heme- d_1 biosynthesis)⁵⁷. A knockout mutant in the TatC component of the Tat translocon synthesizes a semi-apocytochrome cd_1 without the heme- d_1 cofactor.

The necessary steps in the biosynthesis of heme d_1 parting from precorrin-2 have been discussed previously¹⁵⁹. They remain still largely unsolved in terms of reactions, underlying enzymes, and correlation with the

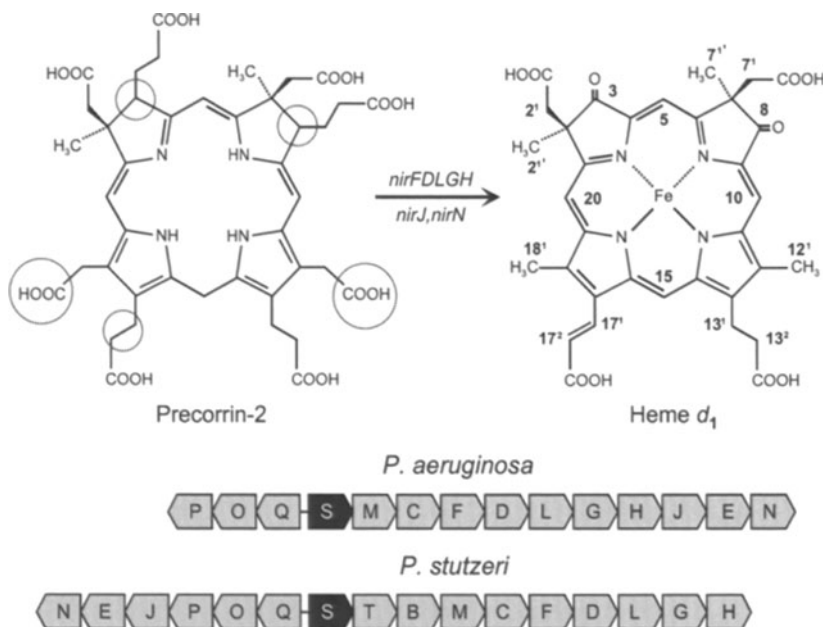


Figure 5. Precursor and product of heme- d_1 biosynthesis. The sites of precorrin-2 that have to be modified, with exception of the desaturation step, are circled. Intermediates in the biosynthetic pathway as well as functions and sequence of action of *nir* gene products have to be established. Bottom part, *nir* gene clusters of pseudomonads harboring the functions for the biosynthesis of heme d_1 and maturation of cytochrome cd_1 . Homologous genes carry the same designations. The structural gene for cytochrome cd_1 , *nirS*, is shown in black. Several *nir* genes are presumed to function in reductase assembly. Heme- d_1 synthesis is anticipated to be regulated anaerobically. Recognition motifs for a Crp-Fnr regulator sensing NO or anoxic conditions are found in the promoter regions of *nirE* and the *nirM* operon (*nirMCFDLGH*) of *P. stutzeri* and the *nirS* operon (*nirSMCFDLGHJEN*) of *P. aeruginosa*. For further explanation see the text.

nir gene products. The heme- d_1 pathway is assumed to comprise as intermediates precorrin-2 (dihydrosirohydrochlorin) and sirohydrochlorin. The *nirE* gene, which is part of the *nir* clusters (Figure 5), encodes an *S*-adenosyl-L-methionine-dependent uroporphyrinogen III methyltransferase (EC 2.1.1.107). Mutagenesis of *nirE* results in a semi-apocytochrome cd_1 that lacks heme d_1 ³⁴. One can assume that NirE activity yields precorrin-2. Dehydrogenation of this intermediate results in sirohydrochlorin.

In the branch of siroheme synthesis of *E. coli* the entire pathway from uroporphyrinogen III to siroheme is catalyzed by a multifunctional enzyme encoded by the *cysG* locus¹⁴¹. The NirE proteins of *P. stutzeri*¹⁵⁹ and *P. aeruginosa*⁶⁹ are homologous to CysG, but lack its N-terminal domain which catalyzes dehydrogenation and Fe chelation. Analogous to CysG, the

nirF gene product has been initially claimed to be responsible for heme- d_1 synthesis in *P. aeruginosa*⁶⁸, but clearly a single protein is insufficient to accomplish the multiple steps necessary to convert precorrin-2 to heme d_1 . This transformation requires at least the products of the entire *nirD* locus, *nirFDLGH* (Figure 5), as demonstrated with *P. stutzeri*⁹⁶. Mutations in this locus and several other *nir* genes result in the absence of heme d_1 from the enzyme or in a dysfunctional cytochrome cd_1 ^{34, 69, 96, 150, 162}.

In addition to the enzymes for the synthesis of the macrocycle there is mutational evidence for further functions necessary for the assembly or maturation of cytochrome cd_1 . Mutation of the *nirJ* and *nirN* genes of *P. aeruginosa* result in a Nir⁻ phenotype without preventing NirS synthesis⁶⁹. NirJ is similar to proteins of biosynthetic pathways for pyrroloquinoline quinone (PqqE/PqqIII/Pqq), the Mo cofactor of nitrogenase (NifB), and molybdopterin (MoaA). Like NirE, NirJ is a member of the SAM family of *S*-adenosyl methionine-dependent enzymes utilizing a radical mechanism¹²⁴. The NirS protein synthesized in a *nirJ* mutant is reactivated in cell extract by adding heme d_1 ⁶⁹. Therefore, a distinct maturation step does not proceed in a NirJ⁻ background or the loss of a biosynthetic reaction catalyzed by NirJ leads to a macrocycle intermediate that is not incorporated into apocytochrome cd_1 .

NirN is a heme protein which has similarity with NirS and is related in its heme *c*-binding domain to NirC¹⁵⁹. The soluble protein is located in the periplasm since an 18-amino-acid signal peptide is cleaved from the precursor protein⁵⁵. The *nirN* mutant has even residual *in vivo* activity. NirF shows also similarity with NirS, however, it lacks the heme *c*-binding domain of cytochrome cd_1 or NirN. The cellular location of NirF is unknown. The similarities of NirF and NirN with NirS point to a scaffold function in heme- d_1 synthesis or heme export. A proper heme- d_1 transporter has not been identified but seems required given the evidence from *tat* mutagenesis with *P. stutzeri*, which discards the possibility that a folded apocytochrome cd_1 could serve as carrier for heme d_1 . Finally, the *nirQOP* operon affects maturation of cytochrome cd_1 or its *in vivo* function without preventing synthesis. The *nirO* product is structurally related to the subunit III of cytochrome-*c* oxidase. A *nirQ*⁺ strain *P. aeruginosa*, lacking the *nirQ* operon, has an about 20% decreased growth yield per molar electron consumption⁵.

5.3. Master Regulators of Denitrification

Denitrification is expressed in the absence or at a lowered tension of oxygen when simultaneously an N-oxide is present. Genes encoding denitrification regulators, termed *dnr*, *dnrD*, or *nnrR*, were found on sequencing the regions downstream of the *nor* operons of *P. aeruginosa*⁴, *P. stutzeri*^{137, 159}, and *Pseudomonas* sp. G-179¹¹. The Dnr and NnrR regulators are structural and

functional homologues of the Crp-Fnr superfamily which have evolved into two clearly separate branches⁷². Members of the Dnr branch are Dnr of *P. aeruginosa*, DnrD of *P. stutzeri*, Nnr of *P. denitrificans*, and others (some even termed Fnr), whereas the second branch harbors the NnrR regulators of *Pseudomonas* sp. G-179, *R. sphaeroides*, *Bradyrhizobium japonicum*, and others, and also Dnr of *Alcaligenes faecalis* S-6 and Nnr of *R. sphaeroides* IL106. The confusing nomenclatorial situation is due to historical reasons and asks for being amended.

The NO sensory pathway mediated by the Dnr and NnrR factors is an important element in the formation of the apparatus for nitrite denitrification. They are responsible for maintaining NO homeostasis and for achieving under anaerobic conditions the coordinate expression of the principal enzymes for nitrite and NO metabolism (Figure 6). Knockout mutagenesis of these regulatory genes in *P. aeruginosa* and *P. stutzeri* shows that they are necessary for the transcriptional control of the *nirS*, *norC*, and *nosR* operons.

NnrR controls transcription of *nirK*. The regulator has been investigated nearly exclusively in *R. sphaeroides*¹³⁰. NnrR was shown to be involved in the NO response to establish nitrite respiration in this phototroph. A distinguishing feature of an NnrR factor is a histidine substitution for the glutamate in the E-SR sequence of the DNA-recognition helix. A genuine NnrR factor is associated with the *nirK* gene of *Pseudomonas* sp. G-179¹¹. There seems to be a specificity of Dnr and NnrR regulators acting on the *nirS* and *nirK* promoters, respectively (Figure 6).

All Dnr and NnrR target promoters carry partially palindromic motifs that are similar to the *E. coli* Fnr box, TTGAT-N₄-ATCAA. The center of this

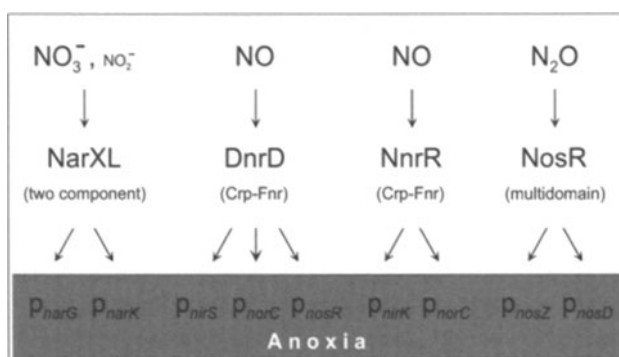


Figure 6. Signal molecules, signal transduction pathways, and regulators of denitrification. The interaction of the signaling gases with the regulators is not proven experimentally and may also be of indirect nature. Lower case “p” denotes a target promoter. For details on the NosR regulator see Section 6.4.1.

regulatory motif is located at -40.5 to -43.5 of the transcript start. Promoters of this type belong to class II where the activator makes contact promoter-proximal to the β -subunit of RNA polymerase, overlapping the -35 promoter element, and promoter-distal with the C-terminal domain of the α -subunit¹⁹. Direct binding of a Dnr factor to target DNA has not been shown, but is strongly suggested from mutational evidence with the *nirS* and *norC* promoters of *P. denitrificans*^{62, 117}. *dnrD* of *P. stutzeri* is organized as a *dnrNODP* operon which is activated in response toward NO ¹³⁹.

The question of how Dnr and NnrR regulators are converted to active transcription factors is not solved. Constitutive overexpression of *dnrD* in *P. stutzeri* is insufficient to activate DnrD-dependent target promoters unless the N-oxide signal is also provided¹³⁹. This argues for the necessity of DnrD activation by NO and the existence of interconvertible active and inactive states. The mechanism of activation seems to be of a general nature, since the homologous denitrification regulator of *P. denitrificans* expressed in *E. coli* can be activated by an NO donor⁶¹.

6. NITROUS OXIDE REDUCTASE AND ASSEMBLY OF ITS COPPER CENTERS

N_2OR transforms nitrous oxide to N_2 as the last step of a complete denitrification process. The soluble, periplasmic enzyme provides an electron sink for energy conservation with proton translocation being associated with the constitutive coupling complexes of the aerobic respiratory chain^{77, 111, 131}. *P. stutzeri* and other denitrifying bacteria can grow with N_2O as the sole electron acceptor, that is, N_2O reduction represents a respiratory process in its own right. *P. aeruginosa* is a complete denitrifier and disposes over all enzymes, but does not grow on exogenous N_2O . This is due to a requirement of NO as the inducer of the *nos* operon and for the synthesis of N_2OR ⁷. Bacteria are also known that respire N_2O without being denitrifiers, substantiating in this way the self-sustaining nature of N_2O respiration^{9, 86, 154}.

6.1. Nitrous Oxide Reductase

N_2OR is a homodimeric Cu-containing protein with two metal centers Cu_A and Cu_Z , representing the electron transfer and catalytic site, respectively. The enzyme exists in several forms distinguished by their redox and spectroscopic properties. The purple species or type I reductase represents the catalytically most active form of *P. stutzeri*. Form I exhibits in the resting state an absorbance maximum around 540 nm with a shoulder at 480 nm, a low intensity band at 350 nm, and a broad band around 780 nm. On addition of

dithionite the enzyme turns blue and becomes catalytically incompetent (form III). In contrast a blue enzyme from *P. nautica* resulting from an aerobic isolation procedure is catalytically active. It is this form of which the first crystal structure of an N₂OR has been obtained¹⁷.

The N-terminal domain comprises about 450 amino acids and forms a seven-bladed β -propeller (Figure 7A). The catalytic site, Cu_Z, resides at the solvent-oriented site of the central channel of this propeller. The C-terminal domain carries Cu_A and has the cupredoxin fold of azurins. The domain consists in about 130 amino acids and is joined with the catalytic domain by a linker peptide of low positional conservation²². A protein monomer harbors in addition to six Cu atoms two calcium atoms and a chloride ion¹⁷. The calcium atoms are involved in dimer formation together with a number of Cu-binding residues at the monomer–monomer interface⁵⁴. The two subunits are tightly coupled because of domain exchange between them. The latter brings the two

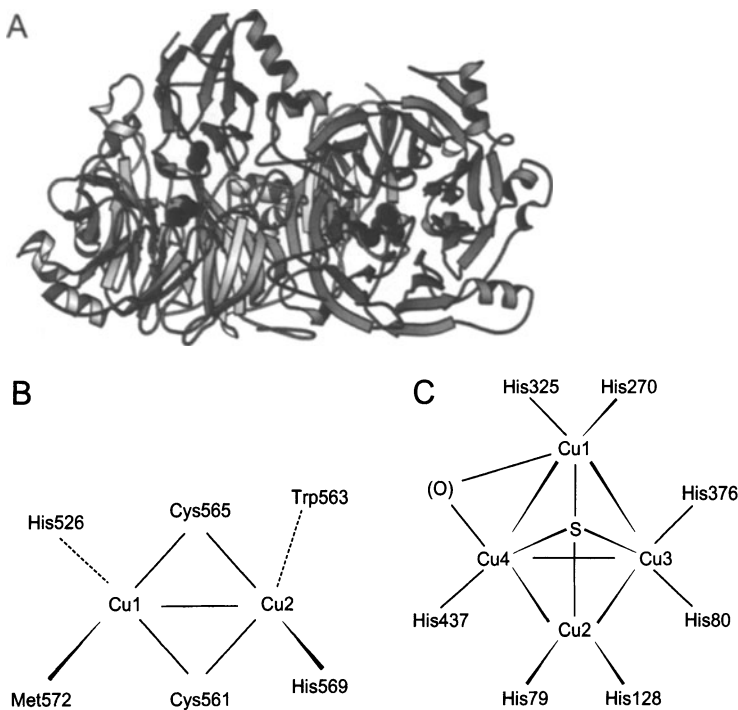


Figure 7. Structures of nitrous oxide reductase and its Cu centers. (A) Crystal structure of N₂OR from *P. nautica*. Atomic coordinates are from the Protein Data Bank file 1QNI¹⁸; the structure is drawn with MolScript v2.1⁷⁴. Locations of the calcium and chloride ions are not shown. (B) Structure of the binuclear Cu_A site representing the electron entry site. (C) The tetranuclear Cu_Z center representing the catalytic site.

Cu centers of the enzyme within ~ 10 Å, which is much closer than their intra-subunit distance of ~ 40 Å. Such a large distance would be unsuitable for rapid intramolecular electron transfer.

6.2. Cu_A, a New Type of Copper Center in Proteins

The nature of the Cu_A center has been correctly deduced from a combination of biochemical, spectroscopic, and genetic approaches (Figure 7B) (evidence summarized in ref. [22]). The candidate ligands of the Cu_A center were identified from site-directed mutagenesis of N₂OR. They correspond to the two cysteines, two histidines, and one methionine predicted as ligands from comparative sequence analysis of N₂OR and the subunit II of cytochrome-*c* oxidase. Whether or not the Cu pair in the Cu_A center would be bridged by the cysteines, remained open until a crystal structure of a Cu_A protein was obtained, which resolved this question in favor of a doubly cysteine-bridged structure¹⁴⁵. The crystal structure also revealed a carbonyl group from a main peptide chain glutamate as a Cu_A ligand, which is provided by a tryptophan in N₂OR. The Cu_A ligands are conserved in the primary structure of N₂O reductases and cytochrome-*c* oxidases, spanning biology from prokaryotes to man. The hyperfine structure of the EPR spectrum of N₂OR is well resolved both at low and high field and is the result of a mixed-valence interaction of two neighboring Cu atoms⁷⁶. The unraveling of the binuclearity of Cu_A of N₂OR by spectroscopic techniques has been a key element for recognizing the same structure in cytochrome-*c* oxidase^{2, 12}.

6.3. Cu_Z, the First Biologically Active Cu–S Center

The basic structure of the catalytic center, Cu_Z, is a μ_4 -sulfide-bridged tetranuclear Cu cluster (Figure 7C). The Cu–S bond lengths are all around 2.3 Å; the Cu1–S–Cu2 angle is about 161°. The Cu–Cu distances are slightly unequal with Cu1 being more distant from Cu atoms 3 and 4, than Cu2 from its neighboring Cu atoms. The primary structures of N₂OR from different sources show a high degree of conservation particularly around the ligands required for binding the two Cu centers. A remarkable conservation of multiple histidine residues was observed in the first feasible sequence comparison of N₂OR¹⁶³. From a later alignment of eight sequences five conserved regions were filtered out, which carried eight conserved histidines (positions 129, 130, 132, 178, 326, 382, 433, and 494 of *P. stutzeri*). This indicated the contribution of multiple histidines to the coordination of Cu_Z. Further it was suggested from site-directed mutagenesis that His494 is ligand to Cu_Z²².

The crystal structure revealed that all conserved histidine residues, except His132, are indeed ligands of Cu_Z. The equivalent *P. nautica* residue to *P. stutzeri* His494 was shown to be ligand to Cu4¹⁷. Since Cu1 and Cu4 are bridged by a hydroxyl or water molecule^{17, 54}, it is thought that this entity can be replaced by N₂O, to make the Cu1–Cu4 edge the substrate-binding site. N₂O is suggested to be oriented with the oxygen atom toward Cu1. The purpose of the Cu₄–S cluster is to facilitate a two-electron reduction of N₂O. One electron is derived from Cu4, whereas the other comes from Cu2 via a Cu2–S–Cu1 σ/σ superexchange pathway²⁴.

Because of its unprecedented multinuclear nature, spectroscopic methods were not equally successful in predicting the Cu_Z site as compared to Cu_A. However, the accumulated spectroscopic evidence for sulfur coordination and, at the very end, chemical analysis contributed essential findings that led to the elucidation of Cu_Z as the first biologically relevant copper-sulfide cluster^{1, 108}. Resonance Raman and magnetic circular dichroism spectroscopy had indicated a highly covalent Cu thiolate site in form III of N₂OR^{1, 36, 39}. The situation remained puzzling, however, since mutagenesis of the single conserved cysteine outside the Cu_A domain only destabilized but did not inactivate N₂OR³⁸. Also homologous cysteine residues were missing, or would have to be positionally shifted, in the N₂OR sequences of “*Achromobacter cycloclastes*”⁸⁹ and *P. nautica*¹⁰⁶. In the chain of reasoning for a Cu–S site the engineered Cys622Asp mutant of *P. stutzeri* N₂OR proved pivotal. This mutant had lost the Cu_A site due to the substitution of one liganding cysteine and allowed the selective investigation of Cu_Z. The resonance Raman properties of the mutant protein showed a Cu–sulfur interaction, which was proven by ³⁴S-labeling of wild-type N₂OR. Lacking a conserved cysteine for Cu_Z, the enzyme was analyzed for sulfur, which revealed the stoichiometric presence of one inorganic sulfide per enzyme monomer. Inorganic sulfide was likewise detected in N₂OR of *P. pantotrophus*¹⁰⁸. A high-resolution x-ray analysis of N₂OR of *P. denitrificans* established then the Cu₄–S structure which was also confirmed for the *P. nautica* enzyme¹⁷. Initially the x-ray structure of N₂OR had revealed a tetranuclear Cu₄–O cluster as the catalytic site¹⁸. Overall, it lasted nearly two decades from the discovery of the enzyme¹⁶⁶ until the basic structure of the catalytic center of N₂OR was established. Recently it has been pointed out that the blue enzyme form, of which the crystal structure has been obtained, has a modified catalytic center, Cu_Z*¹⁰⁷. Magnetic circular and optical circular dichroic properties of the blue form from dithionite reduction are clearly different from the blue form resulting from aerobic enzyme purification. The structural basis of this aspect of the enzyme is still insufficiently resolved. Cu_Z* forms have been generated also by modifications of the distal liganding histidine, His583, and the bridging cysteine, Cys622, of the Cu_A center²². This and several other observations point to an interaction of the Cu_A and Cu_Z domains which may be responsible for the observed spectral changes.

6.4. Enzyme Maturation

The assembly of the conjugated Cu-S center at the catalytic site has become an intriguing question. N₂OR was among the proteins for which a requirement of ancillary proteins catalyzing metal insertion was deduced early from genetic evidence¹⁶⁷. Important elements in this process are the nature and availability of a metal donor, the site of synthesis of the metal center, and ancillary components for protein folding and metal insertion. Cu homeostasis of the bacterial cell has to balance metal requirement for N₂OR (and other vital Cu proteins) and metal toxicity. Therefore, Cu for N₂OR is processed along strictly regulated pathways, presumably beginning with a porin at the outer membrane, and followed by targeting of Cu to acceptor or chaperone proteins before inserting the metal into the apoenzyme.

N₂OR biosynthesis has been studied mostly with *P. stutzeri*. The enzyme is located in the periplasm where Cu is inserted into the apoenzyme. Evidence for N₂OR location comes from immunogold labeling together with electron microscopy and from cell fractionation. N₂OR is exported by the Tat system; the enzyme precursor thus carries the Tat specific signal peptide with a twin arginine motif⁵⁷. N₂OR, retained in the cytoplasm by mutating an arginine of the signal peptide or by inactivating the principal transport gene, *tatC*, is synthesized in each case as the apoprotein only. That is, the maturation of N₂OR is a post-translocational process.

6.4.1. Components for Cu₂ Assembly

An *in silico* survey reveals a consistent association of *nosZ* with the *nosR* gene. A *nosR* insertion mutant of *P. stutzeri* lacks *nosZ* transcript, which supports a *trans*-acting function for NosR^{32, 139}. The synthesis of NosR is under the control of DnrD, which is consistent with the presence of cognate recognition motifs, although in noncanonical distances to the transcription start site³¹. *nosZ* of *P. stutzeri* has a monocistronic transcript which is expressed from six promoters. Anaerobic expression occurs preferentially from promoter P3, whereas the weak constitutive expression of the enzyme in aerobic cells is likely to depend on promoter P2³¹. While NosR is essential for *nosZ* transcription in *P. stutzeri*, the *nos* gene cluster of *P. aeruginosa* is transcribed under the control of Dnr from the *nosR* promoter⁷.

The NosR protein (81.9 kDa) has the architecture of a transmembrane-functioning or -signaling protein with a modular five-domain structure extending to either side of the cytoplasmic membrane. Almost half of NosR consists of a periplasmic domain of about 400 amino acids. The C-terminal cytoplasmic domain includes two ferredoxin-like [4Fe-4S] cluster-binding motifs and two further potentially metal-binding motifs, CxxxCP, in two cytoplasmic loops^{31, 159}. Elucidating the structure of NosR is an important objective as this will contribute to an understanding of a wider range of physiological systems.

NosR is a member of a multidomain protein family exhibiting the same metal-binding features. A rearranged domain organization is typical for the potential FeS proteins RdxA, RdxB, and CcoG from *R. sphaeroides*^{71, 95, 113}, and FixG from *B. japonicum*¹⁰⁴. RdxA is assumed to have an oxidoreductase function. *rdxB*, *fixG*, and *ccoG* form each part of a four-gene cluster whose products are involved in the biogenesis and/or stability of the *cbb*₃-type oxidase in the respective bacteria. CprC of *Desulfitobacterium dehalogenans*, another member of this family, is involved in expressing components for halorespiration¹²². In *P. denitrificans* the NosR orthologue NirI is required for *nir* gene expression¹¹⁷.

The biosynthesis of N₂OR with a functional Cu_Z center requires an assembly complex encoded by genes *nosDFY* which are located downstream of *nosZ* and form part of the *nosRZDFYL* gene cluster (Figure 2). Topological information is available from *lacZ* fusions which revealed that NosF is a cytoplasmic component³¹. The *nosD* gene encodes a hydrophilic protein with a signal peptide. It belongs to a large family of β -helix proteins with carbohydrate-binding and sugar hydrolase domains²⁶. The functional significance of these features is not clear. The *nosY* product is highly hydrophobic and represents an integral membrane protein with six transmembrane helices and no significant hydrophilic domains. An α -helix content of over 50% is indicated from tertiary structure prediction. The general arrangement at and on both sides of the cytoplasmic membrane of this three-component assembly complex, and the ATPase activity of NosF, which has been demonstrated recently, are similar to bacterial ABC transporters^{31, 60, 165, 167}.

Inactivation of any protein of the NosDFY complex leads to an N₂OR without the Cu–S site and a correspondingly lower Cu content. The fact that NosD, -F, and -Y proteins fulfill essential roles in the maturation process is revealed by studying expression of *nosZ* in the nondenitrifying *P. putida*. When *nosZ* and *nosR* are provided *in trans* to *P. putida*, it synthesizes a Cu_A-only protein. However, when *nosDFY* are coexpressed, an active N₂OR holoenzyme with a fully assembled Cu_Z center is synthesized in the same heterologous background¹⁴⁸. A role for NosDFY in the assembly of the Cu–S cluster is strongly indicated and its function is more likely in the provision of sulfur than of Cu. N₂OR maturation and Cu center assembly is assumed to comprise the following events (Figure 8). Apo-N₂OR is exported prior to and independent of cofactor insertion by the Tat secretion pathway to the periplasm. Pathways for Cu insertion and sulfur donation converge in the periplasm for Cu–S cluster formation to form holo-N₂OR. Sulfur is thought to be provided from a cytoplasmic source through the action of the NosDFY ABC transporter system. Cu_A can be reconstituted into the apoprotein from exogenous Cu, whereas attempts in that direction have failed for Cu_Z which can be rationalized by the lack of a sulfur source. Cu from the medium may pass through NosA or

bifunctional cell components exerting a rescue function. ScoP is a likely ancillary factor for Cu processing¹⁴⁸. The homologous yeast protein, Sco1, is involved in Cu_A assembly of cytochrome-*c* oxidase. In *Bacillus subtilis* its homologue, YmpQ, affects the cytochrome-*c* oxidase but not menaquinol oxidase, thus favoring a role in Cu_A synthesis⁸⁵. Sco1 homologues have a conserved CxxxCP motif and a histidine residue, which are important for Cu binding²⁵. Furthermore, the soluble domain of the Sco1 homologue PrrC from *R. sphaeroides* has thiol-disulfide oxidoreductase activity which can be used for Cu mobilization⁸⁷. Indeed, each of the sequenced genomes of *nosZ*-harboring bacteria carry a Sco1 homologue.

The *nosX* gene is necessary for N₂O utilization by *Sinorhizobium meliloti*²¹ and *P. denitrificans*¹¹⁶, but is not part of *nos* clusters of *P. stutzeri* and *P. aeruginosa* (Figure 2). It has been suggested that the NosX phenotype is an altered or missing Cu_A center. NosX proteins share limited sequence similarity with RnfF²¹ which, in turn, shows sequence similarity with the lipoprotein ApbE. The *rnfF* gene complements an *apbE* mutant of *Salmonella enterica*¹⁰, which suggests that NosX and ApbE may be functionally interchangeable members of the same protein family. This then could explain a substitute function for *nosX* in the pseudomonads. ApbE has a function in FeS cluster metabolism directed at ThiH which is a putative FeS protein involved in the biosynthesis of thiamine¹²¹. The NosX protein is predicted to be periplasmic because of a signal sequence with features for Tat targeting¹¹⁶.

A current total of 16 individually analyzed denitrifiers and entire genomes show in each case a conserved *nosDFYL* sequence¹⁴⁸. *nosL* is co-transcribed with the assembly genes in *P. stutzeri* and *P. aeruginosa*, which favors for the *nosL* gene product a function related to N₂OR maturation. NosL has the features of a lipoprotein of the outer membrane³⁸. It was purified from "*A. cycloclastes*" as a Cu-containing protein and may provide a metallochaperone role with the putative function to deliver Cu to N₂OR⁸⁸. NosL binds specifically and stoichiometrically one Cu(I) atom per molecule. The Cu(I) site is remarkably stable to oxygen, while the Cu(II) form of NosL has little affinity for Cu and releases the metal. Nonetheless, NosL is not obligatory for N₂OR maturation. NosL proved to be a nonselectable marker and an engineered *nosL* mutant of *P. stutzeri* lacks a recognizable phenotype³⁸.

A further maturation component was identified in *P. stutzeri* JM300 in the form of the outer membrane protein NosA. It was initially thought that NosA inserts Cu into N₂OR, but rather it may be part of a Cu-ion or Cu-chelator uptake system⁸². The repression of *nosA* by 5 µM Cu is indicative of such a function. NosA has a high content of hydrophobic amino acids which may form β-sheets traversing the membrane as a pore. Certain *nosA* mutations are remedied by exogenous Cu, which hints at a further component acting in concert with NosA. The homologue of NosA in *P. aeruginosa* is OprC with 65%

sequence identity¹⁵³. The *oprC* gene maps at a locus not linked to the denitrification gene clusters¹³⁸. NosA/OprC proteins form voltage-gated channels with a slight preference for Cu and have been reported to bind 1–3 Cu atoms. The spectral properties of these proteins and mode of Cu binding are not known. A specific role for NosA in N₂OR biosynthesis could not be established¹⁴⁸. NosA homologues are present in the nondenitrifying bacteria *Yersinia pestis* CO92, *P. putida* KT2440, and *P. fluorescens* PfO-1 arguing against a solely N₂OR-related function.

7. VARIATIONS ON THE *PSEUDOMONAS* THEME

The principal aspects of denitrification described here for *P. stutzeri* and *P. aeruginosa* are to a large extent valid for other cytochrome *cd*₁-carrying denitrifiers. The denitrification system of *P. denitrificans* has been equally well investigated and contributed important advances to the field⁸. A difference between the two genera may exist in the onset of denitrification which seems not to depend in *P. denitrificans* on a NarXL two-component regulatory system but rather on the nitrate-responsive Crp-Fnr regulator, NarR¹⁴⁶. Although *P. aureofaciens* and *Pseudomonas* sp. G-179 are representatives with Cu-containing nitrite reductases, the model organism for this variant of denitrification has been *R. sphaeroides*.

The cytochrome *cb*-type NO reductase shows outside of the pseudomonads two variations in its electron acceptor domain. Remarkably, *Bacillus azotoformans* carries a Cu_A center¹²⁷ which brings the number of known Cu_A-carrying enzymes to three. *R. eutropha* has a monomeric quinol-dependent NO reductase which is under the control of an NtrC-type regulator¹⁰¹. NO reductases show up also in nondenitrifying bacteria, which asks for a physiological explanation for their presence and identification of the source of NO. An important structural variant is known for N₂OR from *Wolinella succinogenes* where a heme domain is fused to the enzyme¹⁵⁶. However, in spite of all these modifications the catalytic domains of the respective enzyme groups exhibit the same basic structure.

In the rhizobia, oxygen-sensing for denitrification expression is exerted by the heme-based FixLJ two-component regulatory system and, in addition, involves regulators belonging to the Crp-Fnr superfamily⁹⁰. Another interesting aspect is nitrate taxis of denitrifying bacteria, which is affected in *R. sphaeroides* by a heme-copper protein encoded within the denitrification gene cluster. Nitrite reductase expression in this bacterium requires not only the NO-responsive regulator, NnrR, but also the two-component PrrBA system, which functions otherwise in the expression of the photosynthetic apparatus^{80, 81}. Since much of the biochemical, structural, and genetic basis

of denitrification is known now in several model organisms, the field is broadening at the organismic side and is shifting more and more to aspects of how this anaerobic respiratory system is embedded in the metabolic and regulatory network of the cell.

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REFERENCES

1. Alvarez, M.L., Ai, J.Y., Zumft, W., Sanders-Loehr, J., and Dooley, D.M., 2001, Characterization of the copper-sulfur chromophores in nitrous oxide reductase by resonance Raman spectroscopy: Evidence for sulfur coordination in the catalytic cluster. *J. Am. Chem. Soc.*, 123:576–587.
2. Antholine, W.E., Kastrau, D.H.W., Steffens, G.C.M., Buse, G., Zumft, W.G., and Kroneck, P.M.H., 1992, A comparative EPR investigation of the multicopper proteins nitrous-oxide reductase and cytochrome *c* oxidase. *Eur. J. Biochem.*, 209:875–881.
3. Arai, H., Igarashi, Y., and Kodama, T., 1994, Structure and ANR-dependent transcription of the *nir* genes for denitrification from *Pseudomonas aeruginosa*. *Biosci. Biotechnol. Biochem.*, 58:1286–1291.
4. Arai, H., Igarashi, Y., and Kodama, T., 1995, Expression of the *nir* and *nor* genes for denitrification of *Pseudomonas aeruginosa* requires a novel CRP/FNR-related transcriptional regulator, DNR, in addition to ANR. *FEBS Lett.*, 371:73–76.
5. Arai, H., Kodama, T., and Igarashi, Y., 1998, The role of the *nirQOP* genes in energy conservation during anaerobic growth of *Pseudomonas aeruginosa*. *Biosci. Biotechnol. Biochem.*, 62:1995–1999.
6. Arai, H., Kodama, T., and Igarashi, Y., 1999, Effect of nitrogen oxides on expression of the *nir* and *nor* genes for denitrification in *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.*, 170:19–24.
7. Arai, H., Mizutani, M., and Igarashi, Y., 2003, Transcriptional regulation of the *nos* genes for nitrous oxide reductase in *Pseudomonas aeruginosa*. *Microbiology*, 149:29–36.
8. Baker, S.C., Ferguson, S.J., Ludwig, B., Page, M.D., Richter, O.-M.H., and van Spanning, R.J.M., 1998, Molecular genetics of the genus *Paracoccus*: Metabolic versatile bacteria with bioenergetic flexibility. *Microbiol. Mol. Biol. Rev.*, 62:1046–1078.
9. Bazyliński, D.A., Frankel, R.B., and Jannasch, H.W., 1988, Anaerobic magnetite production by a marine, magnetotactic bacterium. *Nature (London)*, 334:518–519.
10. Beck, B.J. and Downs, D.M., 1999, A periplasmic location is essential for the role of the ApbE lipoprotein in thiamine synthesis in *Salmonella typhimurium*. *J. Bacteriol.*, 181:7285–7290.
11. Bedzyk, L., Wang, T., and Ye, R.W., 1999, The periplasmic nitrate reductase in *Pseudomonas* sp. strain G-179 catalyzes the first step of denitrification. *J. Bacteriol.*, 181:2802–2806.
12. Beinert, H., 1997, Copper A of cytochrome *c* oxidase, a novel, long-embattled, biological electron-transfer site. *Eur. J. Biochem.*, 245:521–532.
13. Bennisar, A., Rosselló-Mora, R., Lalucat, J., and Moore, E.R.B., 1996, 16S rRNA gene sequence analysis relative to genomovars of *Pseudomonas stutzeri* and proposal of *Pseudomonas balearica* sp. nov. *Int. J. Syst. Bacteriol.*, 46:200–205.

14. Berks, B.C., Ferguson, S.J., Moir, J.W.B., and Richardson, D.J., 1995, Enzymes and associated electron transport systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions. *Biochim. Biophys. Acta*, 1232:97–173.
15. Blümle, S. and Zumft, W.G., 1991, Respiratory nitrate reductase from denitrifying *Pseudomonas stutzeri*, purification, properties and target of proteolysis. *Biochim. Biophys. Acta*, 1057:102–108.
16. Braun, C. and Zumft, W.G., 1991, Marker exchange of the structural genes for nitric oxide reductase blocks the denitrification pathway of *Pseudomonas stutzeri* at nitric oxide. *J. Biol. Chem.*, 266:22785–22788.
17. Brown, K., Djinicovic-Carugo, K., Haltia, T., Cabrito, I., Saraste, M., Moura, J.J.G., Moura, I., Tegoni, M., and Cambillau, C., 2000, Revisiting the catalytic Cu₂ cluster of nitrous oxide (N₂O) reductase. Evidence of a bridging inorganic sulfur. *J. Biol. Chem.*, 275:41133–41136.
18. Brown, K., Tegoni, M., Prudêncio, M., Pereira, A.S., Besson, S., Moura, J.J., Moura, I., and Cambillau, C., 2000, A novel type of catalytic copper cluster in nitrous oxide reductase. *Nature Struct. Biol.*, 7:191–195.
19. Busby, S. and Ebright, R.H., 1999, Transcription activation by catabolite activator protein (CAP). *J. Mol. Biol.*, 293:199–213.
20. Carlson, C.A., Ferguson, L.P., and Ingraham, J.L., 1982, Properties of dissimilatory nitrate reductase purified from the denitrifier *Pseudomonas aeruginosa*. *J. Bacteriol.*, 151:162–171.
21. Chan, Y.-K., McCormick, W.A., and Watson, R.J., 1997, A new *nos* gene downstream from *nosDFY* is essential for dissimilatory reduction of nitrous oxide by *Rhizobium (Sinorhizobium) meliloti*. *Microbiology*, 143:2817–2824.
22. Charnock, J.M., Dreusch, A., Körner, H., Neese, F., Nelson, J., Kannt, A., Michel, H., Garner, C.D., Kroneck, P.M.H., and Zumft, W.G., 2000, Structural investigations of the Cu_A centre of nitrous oxide reductase from *Pseudomonas stutzeri* by site-directed mutagenesis and X-ray absorption spectroscopy. *Eur. J. Biochem.*, 267:1368–1381.
23. Cheesman, M.R., Zumft, W.G., and Thomson, A.J., 1998, The MCD and EPR of the heme centers of nitric oxide reductase from *Pseudomonas stutzeri*: Evidence that the enzyme is structurally related to the heme-copper oxidases. *Biochemistry*, 37:3994–4000.
24. Chen, P., Cabrito, I., Moura, J.J.G., Moura, I., and Solomon, E.I., 2002, Spectroscopic and electronic structure studies of the μ_4 -sulfide bridged tetranuclear Cu₂ cluster in N₂O reductase: Molecular insight into the catalytic mechanism. *J. Am. Chem. Soc.*, 124:10497–10507.
25. Chinenov, Y.V., 2000, Cytochrome *c* oxidase assembly factors with a thioredoxin fold are conserved among prokaryotes and eukaryotes. *J. Mol. Med.*, 78:239–242.
26. Ciccarelli, F.D., Copley, R.R., Doerks, T., Russell, R.B., and Bork, P., 2002, CASH – a β -helix domain widespread among carbohydrate-binding proteins. *Trends Biochem. Sci.*, 27:59–62.
27. Coyne, M.S., Arunakumari, A., Averill, B.A., and Tiedje, J.M., 1989, Immunological identification and distribution of dissimilatory heme *cd*₁ and nonheme copper nitrite reductases in denitrifying bacteria. *Appl. Environ. Microbiol.*, 55:2924–2931.
28. Cramm, R., Siddiqui, R.A., and Friedrich, B., 1997, Two isofunctional nitric oxide reductases in *Alcaligenes eutrophus* H16. *J. Bacteriol.*, 179:6769–6777.
29. Cutruzzolà, F., 1999, Bacterial nitric oxide synthesis. *Biochim. Biophys. Acta*, 1411:231–249.
30. Cutruzzolà, F., Arese, M., Grasso, S., Bellelli, A., and Brunori, M., 1997, Mutagenesis of nitrite reductase from *Pseudomonas aeruginosa*: Tyrosine-10 in the c heme domain is not involved in catalysis. *FEBS Lett.*, 412:365–369.
31. Cuypers, H., Berghöfer, J., and Zumft, W.G., 1995, Multiple *nosZ* promoters and anaerobic expression of *nos* genes necessary for *Pseudomonas stutzeri* nitrous oxide reductase and assembly of its copper centers. *Biochim. Biophys. Acta*, 1264:183–190.

32. Cuypers, H., Viebrock-Sambale, A., and Zumft, W.G., 1992, NosR, a membrane-bound regulatory component necessary for expression of nitrous oxide reductase in denitrifying *Pseudomonas stutzeri*. *J. Bacteriol.*, 174:5332–5339.
33. Darwin, A.J. and Stewart, V., 1996, The NAR modulon systems: Nitrate and nitrite regulation of anaerobic gene expression. In E.C.C. Lin and A.S. Lynch (ed.), *Regulation of gene expression in Escherichia coli*, pp. 343–359. Chapman & Hall, New York.
34. de Boer, A.P.N., Reijnders, W.N.M., Kuenen, J.G., Stouthamer, A.H., and van Spanning, R.J.M., 1994, Isolation, sequencing and mutational analysis of a gene cluster involved in nitrite reduction in *Paracoccus denitrificans*. *Antonie van Leeuwenhoek*, 66:111–127.
35. Delorme, S., Philippot, L., Edel-Hermann, V., Deulvot, C., Mougel, C., and Lemanceau, P., 2003, Comparative genetic diversity of the *narG*, *nosZ*, and 16S rRNA genes in fluorescent pseudomonads. *Appl. Environ. Microbiol.*, 69:1004–1012.
36. Dooley, D.M., McGuirl, M.A., Rosenzweig, A.C., Landin, J.A., Scott, R.A., Zumft, W.G., Devlin, F., and Stephens, P.J., 1991, Spectroscopic studies of the copper sites in wild-type *Pseudomonas stutzeri* N₂O reductase and in an inactive protein isolated from a mutant deficient in copper-site biosynthesis. *Inorg. Chem.*, 30:3006–3011.
37. Dreusch, A., Bürgisser, D.M., Heizmann, C.W., and Zumft, W.G., 1997, Lack of copper insertion into unprocessed cytoplasmic nitrous oxide reductase generated by an R20D substitution in the arginine consensus motif of the signal peptide. *Biochim. Biophys. Acta*, 1319:311–318.
38. Dreusch, A., Riester, J., Kroneck, P.M.H., and Zumft, W.G., 1996, Mutation of the conserved Cys165 outside the Cu_A domain destabilizes nitrous oxide reductase but maintains its catalytic activity: Evidence for disulfide bridges and a putative disulfide isomerase gene. *Eur. J. Biochem.*, 237:447–453.
39. Farrar, J.A., Thomson, A.J., Cheesman, M.R., Dooley, D.M., and Zumft, W.G., 1991, A model of the copper centres of nitrous oxide reductase (*Pseudomonas stutzeri*), evidence from optical, EPR and MCD spectroscopy. *FEBS Lett.*, 294:11–15.
40. Farver, O., Kroneck, P.M.H., Zumft, W.G., and Pecht, I., 2003, Allosteric control of internal electron transfer in cytochrome *cd*₁ nitrite reductase. *Proc. Natl. Acad. Sci. USA*, 100:7622–7625.
41. Fitz-Gibbon, S.T., Ladner, H., Kim, U.-J., Stetter, K.O., Simon, M.I., and Miller, J.H., 2002, Genome sequence of the hyperthermophilic crenarchaeon *Pyrobaculum aerophilum*. *Proc. Natl. Acad. Sci. USA*, 99:984–989.
42. Forte, E., Urbani, A., Saraste, M., Sarti, P., Brunori, M., and Giuffrè, A., 2001, The cytochrome *cbb*₃ from *Pseudomonas stutzeri* displays nitric oxide reductase activity. *Eur. J. Biochem.*, 268:6486–6490.
43. Fülöp, V., Moir, J.W.B., Ferguson, S.J., and Hajdu, J., 1995, The anatomy of a bifunctional enzyme: Structural basis for reduction of oxygen to water and synthesis of nitric oxide by cytochrome *cd*₁. *Cell*, 81:369–377.
44. Fujiwara, T., Fukumori, Y., and Yamanaka, T., 1992, A novel terminal oxidase, cytochrome *baa*₃ purified from aerobically grown *Pseudomonas aeruginosa*: It shows a clear difference between resting state and pulsed state. *J. Biochem.*, 112:290–298.
45. Galimand, M., Gamper, M., Zimmermann, A., and Haas, D., 1991, Positive FNR-like control of anaerobic arginine degradation and nitrate respiration in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 173:1598–1606.
46. Gamble, T.N., Betlach, M.R., and Tiedje, J.M., 1977, Numerically dominant denitrifying bacteria from world soils. *Appl. Environ. Microbiol.*, 33:926–939.
47. Gardner, A.M., Helmick, R.A., and Gardner, P.R., 2002, Flavorubredoxin, an inducible catalyst for nitric oxide reduction and detoxification *Escherichia coli*. *J. Biol. Chem.*, 277:8172–8177.

48. Glockner, A.B., Jüngst, A., and Zumft, W.G., 1993, Copper-containing nitrite reductase from *Pseudomonas aureofaciens* is functional in a mutationally cytochrome *cd₁*-free background (NirS⁻) of *Pseudomonas stutzeri*. *Arch. Microbiol.*, 160:18–26.
49. Godfrey, C., Greenwood, C., Thomson, A.J., Bray, R.C., and George, G.N., 1984, Electron-paramagnetic-resonance spectroscopy studies on the dissimilatory nitrate reductase from *Pseudomonas aeruginosa*. *Biochem. J.*, 224:601–608.
50. Gordon, E.H.J., Sjögren, T., Löfqvist, M., Richter, C.D., Allen, J.W.A., Higham, C.W., Hajdu, J., Fülöp, V., and Ferguson, S.J., 2003, Structure and kinetic properties of *Paracoccus pantotrophus* cytochrome *cd₁* nitrite reductase with the *d₁* heme active site ligand tyrosine 25 replaced by serine. *J. Biol. Chem.*, 278:11773–11781.
51. Grüntzig, V., Nold, S.C., Zhou, J., and Tiedje, J.M., 2001, *Pseudomonas stutzeri* nitrite reductase gene abundance in environmental samples measured by real-time PCR. *Appl. Environ. Microbiol.*, 67:760–768.
52. Härtig, E., Schiek, U., Vollack, K.-U., and Zumft, W.G., 1999, Nitrate and nitrite control of respiratory nitrate reduction in denitrifying *Pseudomonas stutzeri* by a two-component regulatory system homologous to NarXL of *Escherichia coli*. *J. Bacteriol.*, 181:3658–3665.
53. Härtig, E. and Zumft, W.G., 1999, Kinetics of *nirS* expression (cytochrome *cd₁* nitrite reductase) in *Pseudomonas stutzeri* during the transition from aerobic respiration to denitrification: Evidence for a denitrification-specific nitrate- and nitrite-responsive regulatory system. *J. Bacteriol.*, 181:161–166.
54. Haltia, T., Brown, K., Tegoni, M., Cambillau, C., Saraste, M., Mattilas, K., and Djinovic-Carugo, K., 2003, Crystal structure of nitrous oxide reductase from *Paracoccus denitrificans* at 1.6 Å resolution. *Biochem. J.*, 369:77–88.
55. Hasegawa, N., Arai, H., and Igarashi, Y., 2001, Two *c*-type cytochromes, NirM and NirC, encoded in the *nir* gene cluster of *Pseudomonas aeruginosa* act as electron donors for nitrite reductase. *Biochem. Biophys. Res. Commun.*, 288:1223–1230.
56. Hasegawa, N., Arai, H., and Igarashi, Y., 2003, Need for cytochrome *bc₁* complex for dissimilatory nitrite reduction of *Pseudomonas aeruginosa*. *Biosci. Biotechnol. Biochem.*, 67:121–126.
57. Heikkilä, M.P., Honisch, U., Wunsch, P., and Zumft, W.G., 2001, Role of the Tat transport system in nitrous oxide reductase translocation and cytochrome *cd₁* biosynthesis in *Pseudomonas stutzeri*. *J. Bacteriol.*, 183:1663–1671.
58. Heiss, B., Frunzke, K., and Zumft, W.G., 1989, Formation of the N–N bond from nitric oxide by a membrane-bound cytochrome *bc* complex of nitrate-respiring (denitrifying) *Pseudomonas stutzeri*. *J. Bacteriol.*, 171:3288–3297.
59. Hettmann, T., Anemüller, S., Borchering, H., Mathé, L., Steinrücke, P., and Diekmann, S., 2003, *Pseudomonas stutzeri* soluble nitrate reductase $\alpha\beta$ -subunit is a soluble enzyme with a similar electronic structure at the active site as the inner membrane-bound $\alpha\beta\gamma$ holoenzyme. *FEBS Lett.*, 534:143–150.
60. Honisch, U. and Zumft, W.G., 2003, Operon structure and regulation of the *nos* gene region of *Pseudomonas stutzeri*, encoding an ABC-type ATPase for maturation of nitrous oxide reductase. *J. Bacteriol.*, 185:1895–1902.
61. Hutchings, M.I., Shearer, N., Wastell, S., van Spanning, R.J.M., and Spiro, S., 2000, Heterologous NNR-mediated nitric oxide signaling in *Escherichia coli*. *J. Bacteriol.*, 182:6434–6439.
62. Hutchings, M.I. and Spiro, S., 2000, The nitric oxide regulated *nor* promoter of *Paracoccus denitrificans*. *Microbiology*, 146:2635–2641.
63. Ichiki, H., Tanaka, Y., Mochizuki, K., Yoshimatsu, K., Sakurai, T., and Fujiwara, T., 2001, Purification, characterization, and genetic analysis of Cu-containing dissimilatory nitrite reductase from a denitrifying halophilic archaeon, *Haloarcula marismortui*. *J. Bacteriol.*, 183:4149–4156.

64. Inatomi, K.-I. and Hochstein, L.I., 1996, The purification and properties of a copper nitrite reductase from *Haloferax denitrificans*. *Curr. Microbiol.*, 32:72–76.
65. Ishizuka, M., Toraya, T., and Fukui, S., 1984, Purification, properties and limited proteolysis of nitrate reductase from *Pseudomonas denitrificans*. *Biochim. Biophys. Acta*, 786:133–143.
66. Jüngst, A., Wakabayashi, S., Matsubara, H., and Zumft, W.G., 1991, The *nirSTBM* region coding for cytochrome *cd₁*-dependent nitrite respiration of *Pseudomonas stutzeri* consists of a cluster of mono-, di-, and tetraheme proteins. *FEBS Lett.*, 279:205–209.
67. Kalkowski, I. and Conrad, R., 1991, Metabolism of nitric oxide in denitrifying *Pseudomonas aeruginosa* and nitrate-respiring *Bacillus cereus*. *FEMS Microbiol. Lett.*, 82:107–111.
68. Kawasaki, S., Arai, H., Igarashi, Y., and Kodama, T., 1995, Sequencing and characterization of the downstream region of the genes encoding nitrite reductase and cytochrome *c-551* (*nirSM*) from *Pseudomonas aeruginosa*: Identification of the gene necessary for biosynthesis of heme *d₁*. *Gene*, 167:87–91.
69. Kawasaki, S., Arai, H., Kodama, T., and Igarashi, Y., 1997, Gene cluster of dissimilatory nitrite reductase (*nir*) from *Pseudomonas aeruginosa*: Sequencing and identification of a locus for heme *d₁* biosynthesis. *J. Bacteriol.*, 179:235–242.
70. Kiley, P.J. and Beinert, H., 1999, Oxygen sensing by the global regulator FNR: The role of the iron-sulfur cluster. *FEMS Microbiol. Rev.*, 22:341–352.
71. Koch, H.G., Winterstein, C., Saribas, A.S., Alben, J.O., and Daldal, F., 2000, Roles of the *ccoGHIS* gene products in the biogenesis of the *cbb₃*-type cytochrome *c* oxidase. *J. Mol. Biol.*, 297:49–65.
72. Körner, H., Sofia, H.J., and Zumft, W.G., 2003, Phylogeny of the bacterial superfamily of Crp-Fnr transcription regulators: Exploiting the metabolic spectrum by controlling alternative gene programs. *FEMS Microbiol. Rev.*, 27:559–592.
73. Körner, H. and Zumft, W.G., 1989, Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of *Pseudomonas stutzeri*. *Appl. Environ. Microbiol.*, 55:1670–1676.
74. Kraulis, P.J., 1991, *MOLSCRIPT*: A program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr.*, 24:946–950.
75. Kroneck, P.M.H. and Abt, D.J., 2002, Molybdenum in nitrate reductase and nitrite oxidoreductase. In A. Siegel and H. Siegel (ed.), *Metal ions in biological systems*, pp. 369–403. Marcel Dekker, Inc., New York.
76. Kroneck, P.M.H., Antholine, W.A., Riester, J., and Zumft, W.G., 1988, The cupric site in nitrous oxide reductase contains a mixed-valence [Cu(II),Cu(I)] binuclear center: A multifrequency electron paramagnetic resonance investigation. *FEBS Lett.*, 242:70–74.
77. Kundu, B. and Nicholas, D.J.D., 1985, Proton translocation during denitrification in *Rhodopseudomonas sphaeroides* f. *denitrificans*. *Arch. Microbiol.*, 140:358–364.
78. Kuypers, M.M.M., Sliekers, A.O., Lavik, G., Schmid, M., Jörgensen, B.B., Kuenen, J.G., Sinninghe Damsté, J.S., Strous, M., and Jetten, M.S.M., 2003, Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. *Nature*, 422:608–611.
79. Kwiatkowski, A.V. and Shapleigh, J.P., 1996, Requirement of nitric oxide for induction of genes whose products are involved in nitric oxide metabolism in *Rhodobacter sphaeroides* 2.4.3. *J. Biol. Chem.*, 271:24382–24388.
80. Laratta, W.P., Choi, P.S., Tosques, I.E., and Shapleigh, J.P., 2002, Involvement of the PrrB/PrrA two-component system in nitrite respiration in *Rhodobacter sphaeroides* 2.4.3: Evidence for transcriptional regulation. *J. Bacteriol.*, 184:3521–3529.
81. Lee, D.Y., Ramos, A., Macomber, L., and Shapleigh, J.P., 2002, Taxis response of various denitrifying bacteria to nitrate and nitrite. *Appl. Environ. Microbiol.*, 68:2140–2147.
82. Lee, H.S., Abdelal, A.H.T., Clark, M.A., and Ingraham, J.L., 1991, Molecular characterization of *nosA*, a *Pseudomonas stutzeri* gene encoding an outer membrane protein required to make copper-containing N₂O reductase. *J. Bacteriol.*, 173:5406–5413.

83. Liu, H.-P., Takio, S., Satoh, T., and Yamamoto, I., 1999, Involvement in denitrification of the *napKEFDABC* genes encoding the periplasmic nitrate reductase system in the denitrifying phototrophic bacterium *Rhodobacter sphaeroides* f. sp. *denitrificans*. *Biosci. Biotechnol. Biochem.*, 63:530–536.
84. Matsushita, K., Shinagawa, E., Adachi, O., and Ameyama, M., 1982, *o*-Type cytochrome oxidase in the membrane of aerobically grown *Pseudomonas aeruginosa*. *FEBS Lett.*, 139:255–258.
85. Mattatall, N.R., Jazairi, J., and Hill, B.C., 2000, Characterization of YpmQ, an accessory protein required for the expression of cytochrome *c* oxidase in *Bacillus subtilis*. *J. Biol. Chem.*, 275:28802–28809.
86. McEwan, A.G., Greenfield, A.J., Wetzstein, H.G., Jackson, J.B., and Ferguson, S.J., 1985, Nitrous oxide reduction by members of the family *Rhodospirillaceae* and the nitrous oxide reductase of *Rhodopseudomonas capsulata*. *J. Bacteriol.*, 164:823–830.
87. McEwan, A.G., Lewin, A., Davy, S.L., Boetzel, R., Leech, A., Walker, D., Wood, T., and Moore, G.R., 2002, PrrC from *Rhodobacter sphaeroides*, a homologue of eukaryotic Sco proteins, is a copper-binding protein and may have a thiol-disulfide oxidoreductase activity. *FEBS Lett.*, 518:10–16.
88. McGuirl, M.A., Bollinger, J.A., Cosper, N., Scott, R.A., and Dooley, D.M., 2001, Expression, purification, and characterization of NosL, a novel Cu(I) protein of the nitrous oxide reductase (*nos*) gene cluster. *J. Biol. Inorg. Chem.*, 6:189–195.
89. McGuirl, M.A., Nelson, L.K., Bollinger, J.A., Chan, Y.-K., and Dooley, D.M., 1998, The *nos* (nitrous oxide reductase) gene cluster from the soil bacterium *Achromobacter cycloclastes*: Cloning, sequence analysis, and expression. *J. Inorg. Biochem.*, 70:155–169.
90. Mesa, S., Bedmar, E., Chanfon, A., Hennecke, H., and Fischer, H.-M., 2003, *Bradyrhizobium japonicum* NnrR, a denitrification regulator, expands the FixLJ-FixK₂ regulatory cascade. *J. Bacteriol.*, 185:3978–3982.
91. Moir, J.W.B. and Wood, N.J., 2001, Nitrate and nitrite transport in bacteria. *Cell. Mol. Life Sci.*, 58:215–224.
92. Moreno-Vivián, C., Cabello, P., Martínez-Luque, M., Blasco, R., and Castillo, F., 1999, Prokaryotic nitrate reduction: Molecular properties and functional distinction among bacterial nitrate reductases. *J. Bacteriol.*, 181:6573–6584.
93. Moura, I. and Moura, J.J.G., 2001, Structural aspects of denitrifying enzymes. *Curr. Opin. Chem. Biol.*, 5:168–175.
94. Nurizzo, D., Silvestrini, M.-C., Mathieu, M., Cutruzzolà, F., Bourgeois, D., Fülöp, V., Hajdu, J., Brunori, M., Tegoni, M., and Cambillau, C., 1997, N-terminal arm exchange is observed in the 2.15 Å crystal structure of oxidized nitrite reductase from *Pseudomonas aeruginosa*. *Structure*, 5:1157–1171.
95. O’Gara, J.P. and Kaplan, S., 1997, Evidence for the role of redox carriers in photosynthesis gene expression and carotenoid biosynthesis in *Rhodobacter sphaeroides* 2.4.1. *J. Bacteriol.*, 179:1951–1961.
96. Palmedo, G., Seither, P., Körner, H., Matthews, J.C., Burkhalter, R.S., Timkovich, R., and Zumft, W.G., 1995, Resolution of the *nirD* locus for heme *d₁* synthesis of cytochrome *cd₁* (respiratory nitrite reductase) from *Pseudomonas stutzeri*. *Eur. J. Biochem.*, 232:737–746.
97. Philippot, L., 2002, Denitrifying genes in bacterial and archaeal genomes. *Biochim. Biophys. Acta*, 1577:355–376.
98. Philippot, L., Clays-Josserand, A., Lensi, R., Trinsoutreau, I., Normand, P., and Potier, P., 1997, Purification of the dissimilative nitrate reductase of *Pseudomonas fluorescens* and the cloning and sequencing of its corresponding genes. *Biochim. Biophys. Acta*, 1350:272–276.
99. Philippot, L., Mirleau, P., Mazurier, S., Siblot, S., Hartmann, A., Lemanceau, P., and Germon, J.C., 2001, Characterization and transcriptional analysis of *Pseudomonas fluorescens* denitrifying clusters containing the *nar*, *nir*, *nor*, and *nos* genes. *Biochim. Biophys. Acta*, 1517:436–440.

100. Pitcher, R.S., Cheesman, M.R., and Watmough, N.J., 2002, Molecular and spectroscopic analysis of the cytochrome *cbb₃* oxidase from *Pseudomonas stutzeri*. *J. Biol. Chem.*, 277:31474–31483.
101. Pohlmann, A., Cramm, R., Schmelz, K., and Friedrich, B., 2000, A novel NO-responding regulator controls the reduction of nitric oxide in *Ralstonia eutropha*. *Mol. Microbiol.*, 38:626–638.
102. Poole, R.K. and Hughes, M.N., 2000, New functions for the ancient globin family: Bacterial responses to nitric oxide and nitrosative stress. *Mol. Microbiol.*, 36:775–783.
103. Potter, L., Angove, H., Richardson, D., and Cole, J., 2001, Nitrate reduction in the periplasm of gram-negative bacteria. *Adv. Microb. Physiol.*, 45:51–112.
104. Preisig, O., Zufferey, R., and Hennecke, H., 1996, The *Bradyrhizobium japonicum* *fixGHIS* genes are required for the formation of the high-affinity *cbb₃*-type cytochrome oxidase. *Arch. Microbiol.*, 165:297–305.
105. Priemé, A., Braker, G., and Tiedje, J.M., 2002, Diversity of nitrite reductase (*nirK* and *nirS*) gene fragments in forested upland and wetland soils. *Appl. Environ. Microbiol.*, 68:1893–1900.
106. Prudêncio, M., Pereira, A.S., Tavares, P., Besson, S., Cabrito, I., Brown, K., Samyn, B., Devreese, B., van Beeumen, J., Rusnak, F., Fauque, G., Moura, J.J.G., Tegoni, M., Cambillau, C., and Moura, I., 2000, Purification, characterization, and preliminary crystallographic study of copper-containing nitrous oxide reductase from *Pseudomonas nautica* 617. *Biochemistry*, 39:3899–3907.
107. Rasmussen, T., Berks, B.C., Butt, J.N., and Thomson, A.J., 2002, Multiple forms of the catalytic centre, Cu₂, in the enzyme nitrous oxide reductase from *Paracoccus pantotrophus*. *Biochem. J.*, 364:807–815.
108. Rasmussen, T., Berks, B.C., Sanders-Loehr, J., Dooley, D.M., Zumft, W.G., and Thomson, A.J., 2000, The catalytic center in nitrous oxide reductase, Cu₂, is a copper sulfide cluster. *Biochemistry*, 39:12753–12756.
109. Ray, A. and Williams, H.D., 1996, A mutant of *Pseudomonas aeruginosa* that lacks *c*-type cytochromes has a functional cyanide-insensitive oxidase. *FEMS Microbiol. Lett.*, 135:123–129.
110. Ray, A. and Williams, H.D., 1997, The effects of mutation of the *anr* gene on the aerobic respiratory chain of *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.*, 156:227–232.
111. Richardson, D.J., Bell, L.C., McEwan, A.G., Jackson, J.B., and Ferguson, S.J., 1991, Cytochrome *c₂* is essential for electron transfer to nitrous oxide reductase from physiological substrates in *Rhodobacter capsulatus* and can act as an electron donor to the reductase *in vitro*, correlation with photoinhibition studies. *Eur. J. Biochem.*, 199:677–683.
112. Rösch, C., Mergel, A., and Bothe, H., 2002, Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil. *Appl. Environ. Microbiol.*, 68:3818–3829.
113. Roh, J.H. and Kaplan, S., 2000, Genetic and phenotypic analyses of the *rdx* locus of *Rhodobacter sphaeroides* 2.4.1. *J. Bacteriol.*, 182:3475–3481.
114. Roldán, M.D., Sears, H.J., Cheesman, M.R., Ferguson, S.J., Thomson, A.J., Berks, B.C., and Richardson, D.J., 1998, Spectroscopic characterization of a novel multiheme *c*-type cytochrome widely implicated in bacterial electron transport. *J. Biol. Chem.*, 273:28785–28790.
115. Saraste, M. and Castresana, J., 1994, Cytochrome oxidase evolved by tinkering with denitrification enzymes. *FEBS Lett.*, 341:1–4.
116. Saunders, N.F.W., Hornberg, J.J., Reijnders, W.N.M., Westerhoff, H.V., de Vries, S., and van Spanning, R.J.M., 2000, The NosX and NirX proteins of *Paracoccus denitrificans* are functional homologues: Their role in maturation of nitrous oxide reductase. *J. Bacteriol.*, 182:5211–5217.

117. Saunders, N.F.W., Houben, E.N.G., Koefoed, S., de Weert, S., Reijnders, W.N.M., Westerhoff, H.V., de Boer, A.P.N., and van Spanning, R.J.M., 1999, Transcription regulation of the *nir* gene cluster encoding nitrite reductase of *Paracoccus denitrificans* involves NNR and NirI, a novel type of membrane protein. *Mol. Microbiol.*, 34:24–36.
118. Siddiqui, R.A., Warnecke-Eberz, U., Hengsberger, A., Schneider, B., Kostka, S., and Friedrich, B., 1993, Structure and function of a periplasmic nitrate reductase in *Alcaligenes eutrophus* H16. *J. Bacteriol.*, 175:5867–5876.
119. Silvestrini, M.C., Cutruzzolà, F., D'Alessandro, R., Brunori, M., Fochesato, N., and Zennaro, E., 1992, Expression of *Pseudomonas aeruginosa* nitrite reductase in *Pseudomonas putida* and characterization of the recombinant protein. *Biochem. J.*, 285:661–666.
120. Silvestrini, M.C., Galeotti, C.L., Gervais, M., Schininà, E., Barra, D., Bossa, F., and Brunori, M., 1989, Nitrite reductase from *Pseudomonas aeruginosa*: Sequence of the gene and the protein. *FEBS Lett.*, 254:33–38.
121. Skovran, E. and Downs, D.M., 2003, Lack of the ApbC or ApbE protein results in a defect in Fe-S cluster metabolism in *Salmonella enterica* serovar Typhimurium. *J. Bacteriol.*, 185:98–106.
122. Smidt, H., van Leest, M., van der Oost, J., and de Vos, W.M., 2000, Transcriptional regulation of the *cpr* gene cluster in *ortho*-chlorophenol-respiring *Desulfitobacterium dehalogenans*. *J. Bacteriol.*, 182:5683–5691.
123. Smith, G.B. and Tiedje, J.M., 1992, Isolation and characterization of a nitrite reductase gene and its use as a probe for denitrifying bacteria. *Appl. Environ. Microbiol.*, 58:376–384.
124. Sofia, H.J., Chen, G., Hetzler, B.G., Reyes-Spindola, J.F., and Miller, N.E., 2001, Radical SAM, a novel protein superfamily linking unresolved steps in familiar biosynthetic pathways with radical mechanisms: Functional characterization using new analysis and information visualization methods. *Nucleic Acids Res.*, 29:1097–1106.
125. Spröer, C., Lang, E., Hobeck, P., Burghardt, J., Stackebrandt, E., and Tindall, B.J., 1998, Transfer of *Pseudomonas nautica* to *Marinobacter hydrocarbonoclasticus*. *Int. J. Syst. Bacteriol.*, 48:1445–1448.
126. Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrenner, P., Hickey, M.J., Brinkman, F.S.L., Hufnagle, W.O., Kowalik, D.J., Lagrou, M., Garber, R.L., Goltry, L., Tolentino, E., Westbrook-Wadman, S., Yuan, Y., Brody, L.L., Coulter, S.N., Folger, K.R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G.K.-S., Wu, Z., Paulsen, I.T., Reizer, J., Saier, M.H., Hancock, R.E.W., Lory, S., and Olson, M.V., 2000, Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature*, 406:959–964.
127. Suharti, Strampraad, M.J.F., Schröder, I., and de Vries, S., 2001, A novel copper A containing menaquinol NO reductase from *Bacillus azotoformans*. *Biochemistry*, 40:2632–2639.
128. Thöny-Meyer, L., 1997, Biogenesis of respiratory cytochromes in bacteria. *Microbiol. Mol. Biol. Rev.*, 61:337–376.
129. Tosques, I.E., Kwiatkowski, A.V., Shi, J., and Shapleigh, J.P., 1997, Characterization and regulation of the gene encoding nitrite reductase in *Rhodobacter sphaeroides* 2.4.3. *J. Bacteriol.*, 179:1090–1095.
130. Tosques, I.E., Shi, J., and Shapleigh, J.P., 1996, Cloning and characterization of *nnrR*, whose product is required for the expression of proteins involved in nitric oxide metabolism in *Rhodobacter sphaeroides* 2.4.3. *J. Bacteriol.*, 178:4958–4964.
131. Urata, K., Shimada, K., and Satoh, T., 1983, Proton translocation associated with denitrification in a photodenitrifier, *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*. *Plant Cell Physiol.*, 24:501–508.
132. Urbani, A., Gemeinhardt, S., Warne, A., and Saraste, M., 2001, Properties of the detergent solubilised cytochrome *c* oxidase (cytochrome *ccb₃*) purified from *Pseudomonas stutzeri*. *FEBS Lett.*, 508:29–35.

133. van der Oost, J., de Boer, A.P.N., de Gier, J.-W.L., Zumft, W.G., Stouthamer, A.H., and van Spanning, R.J.M., 1994, The heme-copper oxidase family consists of three distinct types of terminal oxidases and is related to nitric oxide reductase. *FEMS Microbiol. Lett.*, 121:1–10.
134. van Spanning, R.J.M., de Boer, A.P.N., Reijnders, W.N.M., Westerhoff, H.V., Stouthamer, A.H., and van der Oost, J., 1997, FnrP and NNR of *Paracoccus denitrificans* are both members of the FNR family of transcriptional activators but have distinct roles in respiratory adaptation in response to oxygen limitation. *Mol. Microbiol.*, 23:893–907.
135. Viebrock, A. and Zumft, W.G., 1988, Molecular cloning, heterologous expression, and primary structure of the structural gene for the copper enzyme nitrous oxide reductase from denitrifying *Pseudomonas stutzeri*. *J. Bacteriol.*, 170:4658–4668.
136. Vijgenboom, E., Busch, J.E., and Canters, G.W., 1997, *In vivo* studies disprove an obligatory role of azurin in denitrification in *Pseudomonas aeruginosa* and show that *azu* expression is under control of RpoS and ANR. *Microbiology*, 143:2853–2863.
137. Vollack, K.-U., Härtig, E., Körner, H., and Zumft, W.G., 1999, Multiple transcription factors of the FNR family in denitrifying *Pseudomonas stutzeri*: Characterization of four *fnr*-like genes, regulatory responses and cognate metabolic processes. *Mol. Microbiol.*, 31:1681–1694.
138. Vollack, K.-U., Xie, J., Härtig, E., Römling, U., and Zumft, W.G., 1998, Localization of denitrification genes on the chromosomal map of *Pseudomonas aeruginosa*. *Microbiology*, 144:441–448.
139. Vollack, K.-U. and Zumft, W.G., 2001, Nitric oxide signaling and transcriptional control of denitrification genes in *Pseudomonas stutzeri*. *J. Bacteriol.*, 183:2516–2526.
140. Wagner, V.E., Bushnell, D., Passador, L., Brooks, A.I., and Iglewski, B.H., 2003, Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: Effects of growth phase and environment. *J. Bacteriol.*, 185:2080–2095.
141. Warren, M.J., Bolt, E.L., Roessner, C.A., Scott, A.I., Spencer, J.B., and Woodcock, S.C., 1994, Gene dissection demonstrates that the *Escherichia coli* *cysG* gene encodes a multi-functional protein. *Biochem. J.*, 302:837–844.
142. Watmough, N.J., Butland, G., Cheesman, M.R., Moir, J.W.B., Richardson, D.J., and Spiro, S., 1999, Nitric oxide in bacteria: Synthesis and consumption. *Biochim. Biophys. Acta*, 1411:456–474.
143. Williams, P.A., Fülöp, V., Garman, E.F., Saunders, N.F.W., Ferguson, S.J., and Hajdu, J., 1997, Haem-ligand switching during catalysis in crystals of a nitrogen-cycle enzyme. *Nature*, 389:406–412.
144. Williams, P.A., Fülöp, V., Leung, Y.-C., Chan, C., Moir, J.W.B., Howlett, G., Ferguson, S.J., Radford, S.E., and Hajdu, J., 1995, Pseudospecific docking surfaces on electron transfer proteins as illustrated by pseudoazurin, cytochrome *c*₅₅₀ and cytochrome *cd*₁ nitrite reductase. *Nature Struct. Biol.*, 2:975–982.
145. Wilmanns, M., Lappalainen, P., Kelly, M., Sauer-Eriksson, E., and Saraste, M., 1995, Crystal structure of the membrane-exposed domain from a respiratory quinol oxidase complex with an engineered dinuclear copper center. *Proc. Natl. Acad. Sci. USA*, 92:11955–11959.
146. Wood, N.J., Alizadeh, T., Bennett, S., Pearce, J., Ferguson, S.J., Richardson, D.J., and Moir, J.W.B., 2001, Maximal expression of membrane-bound nitrate reductase in *Paracoccus* is induced by nitrate via a third FNR-like regulator named NarR. *J. Bacteriol.*, 183:3606–3613.
147. Wu, W. and Chang, C.K., 1987, Structure of “dione-heme.” Total synthesis of the green heme prosthetic group in cytochrome *cd*₁ dissimilatory nitrite reductase. *J. Am. Chem. Soc.*, 109:3149–3150.
148. Wunsch, P., Herb, M., Wieland, H., Schiek, U.M., and Zumft, W.G., 2003, Requirements for Cu_A and Cu-S center assembly of nitrous oxide reductase deduced from complete periplasmic enzyme maturation in the nondenitrifier *Pseudomonas putida*. *J. Bacteriol.*, 185:887–896.

149. Yamanaka, T., ed., 1992, *The Biochemistry of Bacterial Cytochromes*, Springer-Verlag KG, Berlin, Germany.
150. Ye, R.W., Arunakumari, A., Averill, B.A., and Tiedje, J.M., 1992, Mutants of *Pseudomonas fluorescens* deficient in dissimilatory nitrite reduction are also altered in nitric oxide reduction. *J. Bacteriol.*, 174:2560–2564.
151. Ye, R.W., Fries, M.R., Bezborodnikov, S.G., Averill, B.A., and Tiedje, J.M., 1993, Characterization of the structural gene encoding a copper-containing nitrite reductase and homology of this gene to DNA of other denitrifiers. *Appl. Environ. Microbiol.*, 59:250–254.
152. Ye, R.W., Haas, D., Ka, J.-O., Krishnapillai, V., Zimmermann, A., Baird, C., and Tiedje, J.M., 1995, Anaerobic activation of the entire denitrification pathway in *Pseudomonas aeruginosa* requires Anr, an analog of Fnr. *J. Bacteriol.*, 177:3606–3609.
153. Yoneyama, H. and Nakae, T., 1996, Protein C (OprC) of the outer membrane of *Pseudomonas aeruginosa* is a copper-regulated channel protein. *Microbiology*, 142:2137–2144.
154. Yoshinari, T., 1980, N₂O reduction by *Vibrio succinogenes*. *Appl. Environ. Microbiol.*, 39:81–84.
155. Zannoni, D., 1989, The respiratory chains of pathogenic pseudomonads. *Biochim. Biophys. Acta*, 975:299–316.
156. Zhang, C.-S. and Hollocher, T.C., 1993, The reaction of reduced cytochromes *c* with nitrous oxide reductase of *Wolinella succinogenes*. *Biochim. Biophys. Acta*, 1142:253–261.
157. Zumft, W.G., 1992, The denitrifying prokaryotes. In A. Balows, H.G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), *The Prokaryotes. A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications*, pp. 554–582. Springer-Verlag, New York.
158. Zumft, W.G., 1993, The biological role of nitric oxide in bacteria. *Arch. Microbiol.*, 160:253–264.
159. Zumft, W.G., 1997, Cell biology and molecular basis of denitrification. *Microbiol. Mol. Biol. Rev.*, 61:533–616.
160. Zumft, W.G., 2002, Nitric oxide signaling and NO dependent transcriptional control in bacterial denitrification by members of the FNR-CRP regulator family. *J. Mol. Microbiol. Biotechnol.*, 4:277–286.
161. Zumft, W.G., Braun, C., and Cuypers, H., 1994, Nitric oxide reductase from *Pseudomonas stutzeri*: Primary structure and gene organization of a novel bacterial cytochrome *bc* complex. *Eur. J. Biochem.*, 219:481–490.
162. Zumft, W.G., Döhler, K., Körner, H., Löchelt, S., Viebrock, A., and Frunzke, K., 1988, Defects in cytochrome *cd*₁-dependent nitrite respiration of transposon Tn5-induced mutants from *Pseudomonas stutzeri*. *Arch. Microbiol.*, 149:492–498.
163. Zumft, W.G., Dreusch, A., Löchelt, S., Cuypers, H., Friedrich, B., and Schneider, B., 1992, Derived amino acid sequences of the *nosZ* gene (respiratory N₂O reductase) from *Alcaligenes eutrophus*, *Pseudomonas aeruginosa* and *Pseudomonas stutzeri* reveal potential copper-binding residues: Implications for the Cu_A site of N₂O reductase and cytochrome-*c* oxidase. *Eur. J. Biochem.*, 208:31–40.
164. Zumft, W.G., Gotzmann, D.J., and Kroneck, P.M.H., 1987, Type 1, blue copper proteins constitute a respiratory nitrite-reducing system in *Pseudomonas aureofaciens*. *Eur. J. Biochem.*, 168:301–307.
165. Zumft, W.G. and Kroneck, P.M.H., 1996, Metal-center assembly of the bacterial multi-copper enzyme nitrous oxide reductase. *Adv. Inorg. Biochem.*, 11:193–221.
166. Zumft, W.G. and Matsubara, T., 1982, A novel kind of multi-copper protein as terminal oxidoreductase of nitrous oxide respiration in *Pseudomonas perfectomarinus*. *FEBS Lett.*, 148:107–112.
167. Zumft, W.G., Viebrock-Sambale, A., and Braun, C., 1990, Nitrous oxide reductase from denitrifying *Pseudomonas stutzeri*: Genes for copper-processing and properties of the deduced products, including a new member of the family of ATP/GTP-binding proteins. *Eur. J. Biochem.*, 192:591–599.

PHYSIOLOGICAL EVIDENCE FOR RESPIRATION OF TNT BY *PSEUDOMONAS* SP. JLR11

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The aim of this review is to summarize our current understanding of the anaerobic respiration of 2,4,6-trinitrotoluene (TNT) by a pure bacterial culture of *Pseudomonas* sp. JLR11 from a physiological and biochemical point of view. The article consists of four main sections. Section 1 presents background issues related to TNT pollution and a brief description of TNT metabolism by certain strict anaerobes. Section 2 describes a bacterial strain belonging to the genus *Pseudomonas*, isolated for its capability to use TNT as an N-source. This section also deals with enrichment methodologies. Section 3 examines anaerobic respiration as an emerging new biochemical process, and describes details of the physiological system. Section 4 is devoted to biotechnological applications of anaerobic TNT respiration by *Pseudomonas* sp. JLR11 and its role in bioremediation. Some general considerations are also discussed.

1. INTRODUCTION

TNT degradation has attracted the attention of many scientists in the last decade, probably because a number of Environmental Protection Agencies

have declared TNT a pollutant whose removal is a priority. TNT is a major contaminant in many military sites and explosives manufacturing factories, where production and decommissioning operations generate large quantities of this explosive as a waste product²⁵. Much of this waste is deposited in the soil and in unlined lagoons from which it can reach groundwaters through leaching⁵⁴. Nitroaromatic compounds are true xenobiotic chemicals since a few different natural nitroorganic compounds have been described so far. In addition, the number and location of the nitro groups on the TNT ring prevent attack by classical dioxygenases involved in the degradation of aromatic compounds⁴⁷. Furthermore, electrons from the aromatic ring of TNT are removed by the electronegative nitro groups, a process that makes the nucleus electrophilic. The nitro group consists of two different, highly electronegative elements, of which oxygen is even more electronegative than the nitrogen atom; hence the N–O bond is polarized. The partially positive charge of the nitrogen atom combined with its high electronegativity makes the nitro group easily reducible⁴⁵. Reduction of nitro groups on aromatic rings is widely distributed among living organisms; the process often involves the successive addition of two electrons to the nitro group on TNT to yield the corresponding nitroso, hydroxylamino and amino derivatives. TNT is toxic for many prokaryotes and eukaryotes, and it is mutagenic in *Salmonella enterica* serovar Typhimurium^{7, 48, 57–60, 64}. Complete reduction of the nitro group to an amino group seems to decrease the mutagenic effect of the compound^{16, 30}, whereas nitroso and hydroxylaminodinitrotoluenes make nitroaromatic compounds toxic because they react easily to other biological molecules^{7, 8, 13, 31, 35, 48, 57, 59, 61, 64}. Therefore, TNT remediation is needed to clean up contaminated sites and to remove this toxic and potential mutagenic compound. Although a number of *in situ* and *ex situ* studies for the bioremediation of TNT have been reported^{10, 15, 17, 39, 49}, the intrinsic toxicity of TNT can prevent site restoration.

A number of studies have found that mineralization of TNT under aerobic conditions is limited if it occurs at all^{11, 21, 28, 52, 62}. In addition, many aerobic microbes reduce the nitro groups on the aromatic ring to nitroso and hydroxylamino groups, which have a high propensity to react with each other in the presence of oxygen to produce azoxynitrotoluenes³². These azoxynitrotoluenes are recalcitrant to bioremediation. Degradation of TNT under anaerobic conditions has been explored as an alternative approach to remediation^{12, 18, 19, 27, 36, 39, 40, 47, 55}. This process has the potential advantages of rapid reduction at a low redox potential and diminished polymerization reactions due to the absence of oxygen^{32, 39, 47}.

Two genera have been extensively studied because of their anoxic metabolism of TNT: *Clostridium* and *Desulfovibrio*. The ability to reduce TNT anaerobically is a general phenomenon among *Clostridium* species^{5, 22}. In cell suspension assays with clostridia, reduction of the nitro group resulted in the

production of TAT and other products, some of which remain unidentified. Boopathy and Kulpa⁹ and Preuss *et al.*⁴⁴ isolated strains of the genus *Desulfovibrio* that seemed to use TNT as the sole nitrogen source under sulfate reducing conditions. These strains were also suggested to catalyze the complete reduction of TNT to TAT. The authors above hypothesized that the reductive elimination of the amino groups from TAT occurs by a mechanism analogous to the one described by Schnell and Schinck⁵³ for aniline. However, the mechanism by which the amino groups are released, and the fate of ammonium metabolism by these anaerobes, remain elusive. In contrast, Hawari *et al.*³³ proposed TAT to be a dead-end product in anaerobic treatments, since in sludge plants TAT reacted with itself to form its azo derivatives³⁷.

2. ISOLATION OF A *PSEUDOMONAS* STRAIN THAT USES TNT AS THE FINAL ELECTRON ACCEPTOR

One of the problems in the use of strict anaerobes is their relatively slow growth rate compared to aerobic bacteria. An alternative is the use of facultative microorganisms which are versatile in their adaptation to different oxygen conditions and usually grow faster than strict anaerobes if provided anoxic conditions with the appropriate electron acceptors for the respiratory chains. To search for such microorganisms we set up an enrichment method to isolate bacteria able to use TNT under anoxic conditions²⁷. All enrichments were carried out under an argon atmosphere using culture media supplemented with TNT as the sole N-source and with different C-sources. The microorganisms were obtained from different sites including soils polluted with TNT, herbicides and wastewaters from different sources. Samples were incubated for several days at 30°C and diluted 20-fold in the same culture medium. This procedure was repeated several times. Growth during enrichment was followed turbidometrically. We found that the only successful enrichment grew with glucose as the C-source; and the source of microorganisms was water from a wastewater treatment plant in Granada. In general, we observed that a 20-fold dilution of this enrichment culture in M9 minimal medium with glucose as the C-source and TNT as the sole N-source reached a turbidity higher than 0.3 in 3–7 days. This enrichment culture was spread on plates. A single type colony was found and microbial characterization revealed that it belonged to the genus *Pseudomonas*²⁷. Based on microbiological tests, partial sequencing of the gene encoding the 16S rRNA and fingerprinting with species-specific primers based on Repetitive Extragenic Palindromic (REP) sequences, the strain was assigned to the species *Pseudomonas putida*⁶. In addition to TNT, the strain is able to use nitrate, nitrite and ammonium as an N-source under anoxic conditions²⁷. Mass balances with

TNT have revealed that about 80% of the total nitrogen in TNT was incorporated as cell biomass²⁷. Utilization of TNT as an N-source probably involves the removal of the nitro groups and the release of nitrite, which in turn is reduced to ammonium ions. At present there is no evidence for the enzyme(s) involved in TNT denitration. Cell extracts of *P. putida* strain JLR11 grown anoxically in TNT showed nitrite reductase activity (A. Esteve-Núñez, unpublished), which suggests that this enzyme is responsible for the reduction of nitrite to ammonium ions. Ammonium is incorporated into C-skeletons via the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway (Figure 1).

The isolation in our laboratory of three Tn5 mutants of JLR11 unable to grow on TNT as the N-source supports the existence of such a pathway. One mutant was affected in the *in vivo* reduction of nitrite to ammonium, and the other two mutants exhibited knockouts in the small and large subunits of GOGAT (Caballero, Esteve-Núñez and Ramos, unpublished results). The fate of the C in TNT in *P. putida* JLR11 remains to be elucidated. Analysis of the culture supernatants pointed toward the presence of potential pathway intermediates lacking nitro groups, for example, 2-nitro-4-hydroxybenzoic acid, 4-hydroxybenzaldehyde and 4-hydroxybenzoic acid (Figure 1). The conversion of TNT to these compounds requires not only the removal of the nitro groups, but also the oxidation of the lateral methyl group of TNT to the corresponding aldehyde and carboxylic acid. In assays with [¹⁴C]TNT, only 1% of the radioactivity was detected as ¹⁴CO₂ but 45% was associated to trichloroacetic acid-precipitable cell material.

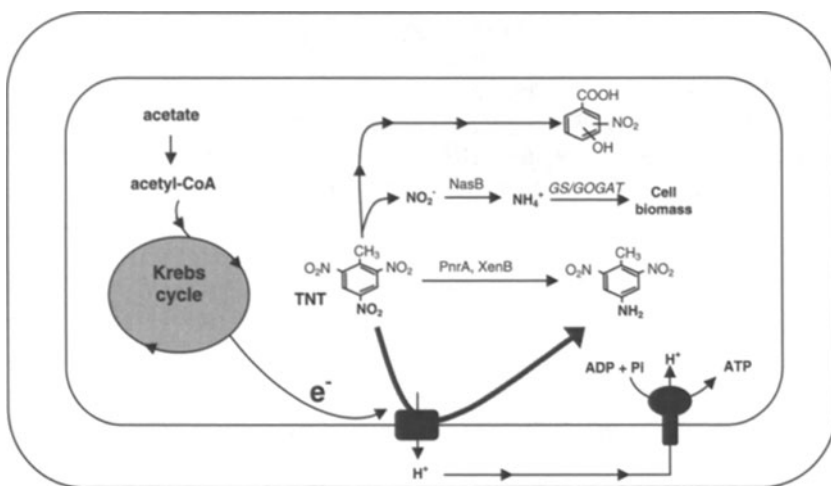


Figure 1. Scheme showing the coupling of electron donor compounds, TNT oxidoreduction, ATP synthesis and assimilation of nitrogen from the TNT ring by *P. putida* JLR11.

3. PHYSIOLOGICAL BASIS FOR
TNT RESPIRATION

The first evidence that TNT can be used as an alternative electron acceptor in respiratory chains came from a series of assays in which different compounds were tested as the sole C-sources²¹. In these assays it was found that *P. putida* strain JLR11 grew on minimal medium with acetate as an electron donor and TNT, regardless of the presence of ammonium ions in the culture medium (Table 1). The oxidation of acetate under anoxic conditions required an electron acceptor, a role that could only be played by TNT in this series of assays. Since strain JLR11 reduced a fraction of the total TNT to monoamino-dinitrotoluenes and diaminomononitrotoluenes, and these products accumulated with time, Esteve-Núñez and Ramos²⁷ hypothesized that the reduced forms of TNT were produced by *Pseudomonas* sp. strain JLR11 when TNT acted as a final electron acceptor in respiratory chains under anoxic conditions (Figure 1). Further support for the role of TNT, other than as an N-source for *Pseudomonas* sp. strain JLR11, came from similar assays but with *Pseudomonas* sp. strain JLR11-P12E2. This mutant, obtained by mini-Tn5-tellurite mutagenesis, was unable to grow on TNT as the sole N-source. The mutant was blocked in the reduction of the released nitrite to ammonium (A. Esteve-Núñez, A. Caballero and J.L. Ramos, unpublished results). However, it was still able to grow under anaerobic conditions with acetate as the sole C-source and ammonium ions as an N-source, but only if TNT was present in the culture medium. Analysis of culture supernatants of this mutant by GC-MS revealed that TNT was mainly reduced to 4-amino-2,6-dinitrotoluene, which provided further evidence for the *in vivo* reduction of

Table 1. Growth of *Pseudomonas* sp. JLR11 and its mutant with acetate and TNT^a.

Strain	TNT	NH ₄ ⁺	Increase in turbidity at 660 nm
JLR11	+	+	0.25–0.3
	+	–	0.22–0.3
	–	+	0
P12E2	+	+	0.25–0.31
	+	–	0
	–	+	0

^aIncreases in turbidity of the cultures under an argon atmosphere with acetate as the C-source and with 5 mM NH₄ Cl and TNT above its saturation level (100 mg/L). Strain P12E2 is an isogenic mutant of the wild-type strain *Pseudomonas* sp. JLR11 that is unable to reduce nitrite to ammonium *in vivo*.

TNT by the mutant cells. These observations suggest that the *para* nitro group in TNT is much more easily reduced than nitro groups *in ortho*. The conversion of a nitro group to an amino group on the TNT ring decreases the electron deficiency of the nitroaromatic ring; consequently, a lower redox potential is required to reduce the rest of the nitro groups of the molecule⁴⁷. Accordingly, only trace amounts of 2,4-diamino-6-nitrotoluene were detected and triaminotoluene was never detected, probably because its formation requires values below -200 mV, which are found only in certain anoxic environments^{29, 34}.

Esteve-Núñez *et al.*²⁶ carried out a series of assays to test whether proton translocation occurred when TNT was added to an anoxic suspension of *Pseudomonas* sp. JLR11. They first induced the respiratory chains involved in TNT respiration by growing cells of *Pseudomonas* sp. strain JLR11 under anoxic conditions on minimal medium with acetate, ammonium and TNT. Washed cell suspensions were incubated in an anoxic isotonic solution of 250 mM sorbitol, and TNT was added to reach 250 μ M. In these cell suspensions Esteve-Núñez *et al.*²⁶ observed a decrease in the pH of the extracellular medium, with maximal acidification after 5 min (Figure 2).

Preincubation of cells with cyanide in the presence of TNT prevented proton extrusion, suggesting that TNT respiration was susceptible to inhibition by cyanide. Esteve-Núñez *et al.*²⁶ also reported that *Pseudomonas* sp. strain JLR11 can use nitrate and nitrite as the final electron acceptor under anoxic

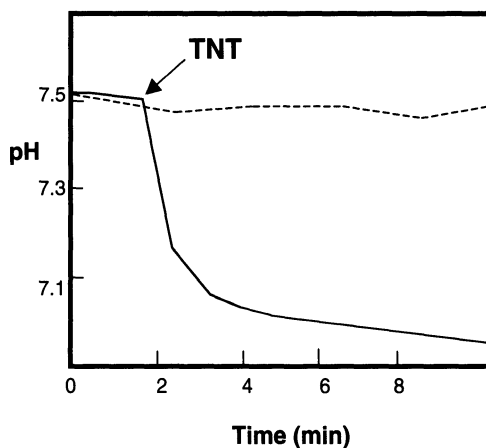


Figure 2. Proton extrusion by *Pseudomonas* sp. JLR11 in response to TNT addition. *Pseudomonas* sp. JLR11 cells were grown under anoxic conditions on M9 minimal medium with TNT as the final electron acceptor²⁶. Washed cells were suspended in sorbitol and split in two halves. When indicated by the arrow TNT was added to one of the halves and changes in the external pH were monitored in time.

conditions; however, while they did note that nitrite- or nitrate-grown cells extruded protons in response to the addition of nitrite and nitrate ($\Delta\text{pH} = -0.4$ unit), the external pH remained unchanged after the addition of TNT. This suggests that nitrate, nitrite respiration and TNT respiration by *Pseudomonas* sp. strain JLR11 are, at least in part, independent processes. Membrane vesicles prepared from nitrate-grown cells failed to reduce TNT. Membrane vesicles prepared from *Pseudomonas* sp. strain JLR11 cells grown with TNT as the electron acceptor catalyzed the reduction of TNT, a process that was accompanied by the oxidation of hydrogen and could be coupled to the synthesis of ATP. No ATP synthesis was observed when the membrane preparation was incubated with gramicidin before the addition of TNT. The above series of findings was interpreted as evidence that different terminal reductases may be involved in processes related to NO_3^- and TNT respiration. Although the studies by Esteve-Núñez *et al.*²⁶ in *Pseudomonas* sp. JLR11 were the first to demonstrate that the reduction of TNT to the corresponding aminonitrotoluenes is physiologically important as an energy conservation system in anoxic conditions, the role of TNT as a potential electron acceptor was first suggested by Boopathy and Kulpa⁹.

4. ENVIRONMENTAL APPLICATIONS AND PERSPECTIVES

The discovery of anaerobic TNT respiration by *P. putida* JLR11 together with the use of TNT as an N-source by this microorganism may not only lead to a better understanding of the physiology, phylogeny and biochemistry of TNT respiring bacteria, but also raises the possibility of interesting environmental applications in anaerobic sites, such as soils and groundwaters, polluted with TNT. In these sites better proliferation of these bacteria is expected because TNT can serve as an N-source, and the TNT respiration process could favor faster growth *in situ*.

Respiration by *P. putida* strain JLR11 is potentially useful for the biotreatment of deep soils polluted with TNT, particularly in phytorhizoremediation, in which bacterial cells are transported to the deepest root zones where oxygen levels are low. Our studies have shown that many *P. putida* strains attach to the surface of seeds and colonize the rhizosphere of a number of plants, reaching cell densities of up to 10^7 colony-forming units per gram of soil^{24, 42}. This is an easy and useful way to deliver high cell densities underground, where oxygen is scarce. Plant-bacterial combinations to phytoremediate contaminated soils were first developed with a *Pseudomonas* sp. strain capable of transforming TNT to its monoaminodinitrotoluene and diaminonitrotoluene metabolites⁵⁶, but the JLR11 strain may turn out to be more

effective and efficient by generating energy through TNT respiration. Current microcosm and field assays are being carried out in our laboratory and should provide a clear view of its potential for *in situ* bioremediation.

The reduced products resulting from TNT respiration can bind strongly to the mineral and organic fractions of the soil, thus immobilizing the amino-nitroaromatic compounds to the soil matrix^{1-4, 14, 17, 18-20, 23, 34, 41}. This type of immobilization has been considered a kind of humification and it is considered a detoxification process because after treatment the reduction metabolites cannot be desorbed from soil by alkaline or acidic hydrolysis or by methanolic extraction. Therefore, it was concluded that metabolites became unavailable for further microbial degradation and mineralization. The covalent binding of reduced metabolites of [¹⁵N]TNT to soil organic matter was analyzed by ¹⁵N nuclear magnetic resonance (NMR) spectroscopy. The NMR shifts showed that nitrogen was covalently bound to humic acid as substituted amines and amides, whereas the NMR spectra of silylated humin suggested the formation of azoxy compounds and imine linkages^{1, 38}. Nonetheless, it should be pointed out that the long-term fate of these immobilized products is unknown.

It has frequently been reported that catabolic genes are part of catabolic plasmids and catabolic transposons^{46, 51, 65}, and it has been shown before that gene exchange, including that of chromosomal markers, can be enhanced in the plant rhizosphere⁵⁰. One would then expect phytorhizoremediation to function by exploiting not only the microbe's own potential but also that of the resident rhizosphere population able to act as recipient of genes for the metabolism of nitroaromatic compounds.

It should be noted that a number of genes involved in TNT metabolism have been described, for example, *xenB*⁴³, *pnrA* (A. Esteve-Núñez, A. Caballero, J.J. Lázaro and J.L. Ramos, unpublished results) and others. These genes can be used as gene probes to identify niches in which these kinds of gene prevail, and the conditions under which the population of microbes bearing these genes increases⁶³. This may also be useful in the design of biological treatments for sites polluted with TNT. *In situ* activities of ongoing biotreatments will be possible by isolating mRNA from cells in polluted sites and monitoring expression with RT-PCR techniques.

Little is known about the biochemical properties of the enzymes involved in TNT metabolism; at present the putative denitrase involved in the removal of the nitro groups from TNT has not been identified, and only a few TNT reductases have been characterized (Esteve-Núñez *et al.*, unpublished results). Therefore we look forward to many important contributions in this research area. The productive steps leading to the mineralization of reduced TNT derivatives by anaerobic bacteria are unknown, and advances are envisaged using microorganisms that can be analyzed with a combination of genetic and biochemical methods. These studies will be illuminating not only in terms of

understanding the catabolic pathways themselves, but also in yielding insights into the evolution of these activities.

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REFERENCES

1. Achtnich, C., Fernandes, E., Bollag, J.M., Knackmuss, H.J., and Lenke, H., 1999, Covalent binding of reduced metabolites of ^{15}N -TNT to soil organic matter during a bioremediation process analyzed by ^{15}N NMR spectroscopy. *Environ. Sci. Technol.*, 33:4448–4456.
2. Achtnich, C. and Lenke, H., 2001, Stability of immobilized 2,4,6-trinitrotoluene metabolites in soil under long-term leaching conditions. *Environ. Toxicol. Chem.*, 20:280–283.
3. Achtnich, C., Lenke, H., Klaus, U., Spiteller, M., and Knackmuss, H.J., 2000, Stability of immobilized TNT derivatives in soil as a function of nitro group reduction. *Environ. Sci. Technol.*, 34:3698–3704.
4. Achtnich, C., Pfortner, P., Weller, M.G., Niessner, R., Lenke, H., and Knackmuss, H.J., 1999, Reductive transformation of bound trinitrophenyl residues and free TNT during a bioremediation process analyzed by immunoassay. *Environ. Sci. Technol.*, 33:3421–3426.
5. Ahmad, F. and Hughes J.B., 2000, Anerobic transformation of TNT by *Clostridium*, In J. Spain, J.N. Hughes, and H.-J. Knackmuss (eds), *Biodegradation of Nitroaromatic Compounds and Explosives*, pp. 185–212. Lewin Publishers, Boca Raton, FL.
6. Aranda-Olmedo, I., Tobes, R., Manzanera, M., Ramos, J.L., and Marqués, S., 2002, Species-specific repetitive extragenic palindromic (REP) sequences in *Pseudomonas putida*. *Nucl. Acids. Res.*, 30:1826–1833.
7. Banerjee, H.N., Verma, M., Hou, L.H., Ashraf, M., and Dutta, S.K., 1999, Cytotoxicity of TNT and its metabolites. *Yale J. Biol. Med.*, 72:1–4.
8. Berthe-Corti, L., Jacobi, H., Kleihauer, S., and White, I., 1998, Cytotoxicity and mutagenicity of a 2,4,6-trinitrotoluene (TNT) and hexogen contaminated soil in *Salmonella typhimurium* and mammalian cells. *Chemosphere*, 37:209–218.
9. Boopathy, R. and Kulpa, C.F., 1992, Trinitrotoluene as a sole nitrogen source for a sulfate-reducing bacterium *Desulfovibrio* sp. (B. strain) isolated from an anaerobic digester. *Curr. Microbiol.*, 25:235–241.
10. Boopathy, R., Manning, J., and Kulpa, C.F., 1997, Optimization of environmental factors for the biological treatment of trinitrotoluene-contaminated soil. *Arch. Environ. Contam. Toxicol.*, 32:94–98.
11. Boopathy, R., Manning, J., and Kulpa, C.F., 1998, A laboratory study of the bioremediation of 2,4,6-trinitrotoluene-contaminated soil using aerobic/anoxic soil slurry reactor. *Water Environ. Res.*, 70:80–86.
12. Boopathy, R., Wilson, M., and Kulpa, C.F., 1993, Anaerobic removal of 2,4,6-trinitrotoluene (TNT) under different electron accepting conditions: Laboratory study. *Water Environ. Res.*, 65:271–275.

13. Brooks, L.R., Jacobson, R.W., Warren, S.H., Kohan, M.J., Donnelly, K.C., and George, S.E., 1997, Mutagenicity of HPLC-fractionated urinary metabolites from 2,4,6-trinitrotoluene-treated Fischer 344 rats. *Environ. Mol. Mutagen.*, 30:298–302, 337.
14. Bruns-Nagel, D., Bretung, J., von Löw, E., Steinbach, K., Borontzy, T., Kahl, M., Blotevogel, K.-H., and Gemsa, D., 1996, Microbial transformation of 2,4,6-trinitrotoluene in aerobic soil columns. *Appl. Environ. Microbiol.*, 62:2651–2656.
15. Bruns-Nagel, D., Steinbach, J.K., Gemsa, D., and von Löw, E., 2000, Composting (humification) of nitroaromatic compounds, In J. Spain, J.B. Hughes, and H.-J. Knackmuss (eds), *Biodegradation of Nitroaromatic Compounds and Explosives*, pp. 357–393. Lewin Publishers, Boca Raton, FL.
16. Cash, G.G., 1998, Prediction of chemical toxicity to aquatic microorganism: ECOSAR vs. Microtox assay. *Environ. Toxicol. Water Qual.*, 132:211–216.
17. Daun, G., Lenke, H., Reuss, M., and Knackmuss, H.J., 1998, Biological treatment of TNT-contaminated soil. 1. Anaerobic cometabolic reduction and interaction of TNT and metabolites with soil components. *Environ. Sci. Technol.*, 32:1956–1963.
18. Drzyzga, D., Bruns-Nagel, D., Gorontzy, T., Blotevogel, K.H., Gemsa, D., and von Löw, E., 1998, Mass balance studies with ¹⁴C-labeled 2,4,6-trinitrotoluene (TNT) mediated by an anaerobic *Desulfovibrio* species and an aerobic *Serratia* species. *Curr. Microbiol.*, 37:380–386.
19. Drzyzga, O., Bruns-Nagel, D., Gorontzy, T., Blotevogel, K.H., Gemsa, D., and von Low, E., 1998, Incorporation of ¹⁴C-labeled 2,4,6-trinitrotoluene metabolites into different soil fractions after anaerobic and anaerobic-aerobic treatment of soil/molasses mixtures. *Environ. Sci. Technol.*, 32:3529–3535.
20. Drzyzga, O., Bruns-Nagel, D., Gorontzy, T., Blotevogel, K.H., and von Löw, E., 1999, Anaerobic incorporation of the radiolabeled explosive TNT and metabolites into the organic soil matrix of contaminated soil after different treatment procedures. *Chemosphere*, 38:2081–2095.
21. Duque, E., Haïdour, A., Godoy, F., and Ramos, J.L., 1993, Construction of a *Pseudomonas* hybrid strain that mineralizes 2,4,6-trinitrotoluene. *J. Bacteriol.*, 175:2278–2283.
22. Ederer, M.M., Lewis, T.A., and Crawford, R.L., 1997, 2,4,6-Trinitrotoluene (TNT) transformation by *Clostridia* isolated from a munition-fed bioreactor: Comparison with non-adapted bacteria. *J. Ind. Microbiol. Biotechnol.*, 18:82–88.
23. Elovitz, M.S. and Weber, E.J., 1999, Sediment-mediated reduction of 2,4,6-trinitrotoluene and fate of the resulting aromatic (poly)amines. *Environ. Sci. Technol.*, 33:2617–2625.
24. Espinosa-Urgel, M., Költner, R., and Ramos, J.L., 2002, Root colonization by *Pseudomonas putida*: Love at first sight. *Microbiology*, 148:341–343.
25. Esteve-Núñez, A., Caballero, A., and Ramos, J.L., 2001, Biological degradation of 2,4,6-trinitrotoluene. *Microbiol. Mol. Biol. Rev.*, 65:335–352.
26. Esteve-Núñez, A., Lucchesi, G., Philipp, B., Schink, B., and Ramos, J.L., 2000, Respiration of 2,4,6-trinitrotoluene by *Pseudomonas* sp. strain JLR11. *J. Bacteriol.*, 182:1352–1355.
27. Esteve-Núñez, A. and Ramos, J.L., 1998, Metabolism of 2,4,6-trinitrotoluene by *Pseudomonas* sp. JLR11. *Environ. Sci. Technol.*, 32:3802–3808.
28. Fernando, T.J., Bumpus J.A., and Aust, S.D., 1990, Biodegradation of TNT (2,4,6-trinitrotoluene) by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.*, 56: 1666–1671.
29. Funk, S.B., Roberts, D.J., Crawford, D.L., and Crawford, R.L., 1993, Initial-phase optimization for bioremediation of munition compound-contaminated soils. *Appl. Environ. Microbiol.*, 59:2171–2177.
30. George, S.E., Huggins-Clark, G., and Brooks, L.R., 2001, Use of a *Salmonella* microsuspension bioassay to detect the mutagenicity of munitions compounds at low concentrations. *Mutat. Res.*, 490:45–56.
31. Gong, P., Wilke, B., and Fleischmann, S., 1999, Soil-based phytotoxicity of 2,4,6-trinitrotoluene (TNT) to terrestrial higher plants. *Arch. Environ. Contam. Toxicol.*, 36:152–157.

32. Haïdour, A. and Ramos, J.L., 1996, Identification of products resulting from the biological reduction of 2,4,6-trinitrotoluene, 2,4-dinitrotoluene, and 2,6-dinitrotoluene by *Pseudomonas* sp. *Environ. Sci. Technol.*, 30:2365–2370.
33. Hawari, J., Halasz, A., Paquet, L., Zhou, E., Spencer, B., Ampleman, G., and Thiboutot, S., 1998, Characterization of metabolites in the biotransformation of 2,4,6-trinitrotoluene with anaerobic sludge: Role of triaminotoluene. *Appl. Environ. Microbiol.*, 64:2200–2206.
34. Hofstetter, T.B., Heijman, C.G., Haderlein, S.B., Holliger, C., and Schwarzenbach, R.P., 1999, Complete reduction of TNT and other (poly)nitroaromatic compounds under iron-reducing subsurface conditions. *Environ. Sci. Technol.*, 33:1479–1487.
35. Honeycutt, M.E., Jarvis, A.S., and McFarland, V.A., 1996, Cytotoxicity and mutagenicity of 2,4,6-trinitrotoluene and its metabolites. *Ecotoxicol. Environ. Saf.*, 35:282–287.
36. Hughes, J.B., Wong, C.H.Y., and Zhang, C.H., 1999, Anaerobic biotransformation of 2,4-dinitrotoluene and 2,6-dinitrotoluene by *Clostridium acetobutylicum*: A pathway through dihydroxylamino intermediates. *Environ. Sci. Technol.*, 33: 1065–1070.
37. Knackmuss, H.-J., 1996, Basis of knowledge and perspectives of bioelimination of xenobiotic compounds. *J. Biotechnol.*, 51:287–295.
38. Knicker, H., Bruns-Nagel, D., Drzyzga, O., von Löw, E., and Steinbach, K., 1999, Characterization of ¹⁵N-TNT residues after an anaerobic/aerobic treatment of soil/molasses mixtures by solid-state ¹⁵N NMR spectroscopy. 1. Determination and optimization of relevant NMR spectroscopy parameters. *Environ. Sci. Technol.*, 33:343–349.
39. Lenke, H., Warrelmann, J., Daun, G., Hund, K., Sieglen, and Knackmuss, H.J., 1998, Biological treatment of TNT-contaminated soil. 2. Biological induced immobilization of the contaminants and full-scale application. *Environ. Sci. Technol.*, 32:1964–1971.
40. Lewis, T.A., Ederer, M.M., Crawford, R.L., and Crawford, D.L., 1997, Microbial transformation of 2,4,6-trinitrotoluene. *J. Ind. Microbiol. Biotechnol.*, 18:89–96.
41. Li, A.Z., Marx, K.A., Walker, J., and Kaplan, D.L., 1997, Trinitrotoluene and metabolites binding to humic acid. *Environ. Sci. Technol.*, 31:584–589.
42. Molina, L., Ramos, C., Duque, E., Ronchel, M.C., García, J.M., Wyke, L., and Ramos, J.L., 2000, Survival of *Pseudomonas putida* KT2440 in soil and in the rhizosphere of plants under greenhouse and environmental conditions. *Soil Biol. Biochem.*, 32:315–321.
43. Pak, J.W., Knoke, K.L., Noguera, D.R., Fox, B.G., and Chambliss, G.H., 2000, Transformation of 2,4,6-trinitrotoluene by purified xenobiotic reductase B from *Pseudomonas fluorescens* I-C. *Appl. Environ. Microbiol.*, 66:4742–4750.
44. Preuss, A., Fimpel, J., and Diekert, G., 1993, Anaerobic transformation of 2,4,6-trinitrotoluene (TNT). *Arch. Microbiol.*, 159:345–353.
45. Preuss, A. and Rieger, P.G., 1995, Anaerobic transformation of 2,4,6-TNT and other nitroaromatic compounds, In J.C. Spain (ed.), *Biodegradation of Nitroaromatic Compounds*, pp. 69–85. Plenum, New York, NY.
46. Ravatn, R., Zehnder, A.J.B., and van der Meer, J.R., 1998, Low-frequency horizontal transfer of an element containing the chlorocatechol degradation genes from *Pseudomonas* sp. strain B13 to *Pseudomonas putida* F1 and to indigenous bacteria in laboratory-scale-activated sludge microorganisms. *Appl. Environ. Microbiol.*, 64:2126–2132.
47. Rieger, P. and Knackmuss, H.J., 1995, Basic knowledge and perspectives on biodegradation of 2,4,6-trinitrotoluene and related nitroaromatic compounds in contaminated soil. In J.C. Spain (ed.), *Biodegradation of Nitroaromatic Compounds*, pp. 1–18. Plenum, New York, NY.
48. Robidoux, P.Y., Hawari, J., Thiboutot, S., Ampleman, G., and Sunahara, G.I., 1999, Acute toxicity of 2,4,6-trinitrotoluene in earthworm (*Eisenia andrei*). *Ecotoxicol. Environ. Saf.*, 44:311–321.
49. Rodgers, J.D. and Bunce, N.J., 2001, Treatment methods for the remediation of nitroaromatic explosives. *Water Res.*, 35:2101–2111.
50. Ronchel, M.C., Ramos-Diaz, M.A., and Ramos, J.L., 2000, Retrotransfer of DNA in the rhizosphere. *Env. Microbiol.*, 2:319–323.

51. Salyers, A.A., Shoemaker, N.B., Stevens, A.M., and Li, L.Y., 1995, Conjugative transposons: An unusual and diverse set of integrated gene transfer elements. *Microbiol. Rev.*, 59:579–590.
52. Scheibner, K., Hofrichter, M., Herre, A., Michels, J., and Fritzsche, W., 1997, Screening for fungi intensively mineralizing 2,4,6-trinitrotoluene. *Appl. Microbiol. Biotechnol.*, 47:452–457.
53. Schnell, S. and Schinck, B., 1991, Anaerobic aniline degradation via reductive deamination of 4-aminobenzoyl-CoA in *Desulfobacterium anilini*. *Arch. Microbiol.*, 155:183–190.
54. Selim, H.M., Xue, S.K., and Iskandar, I.K., 1995, Transport of 2,4,6-trinitrotoluene and hexahydro-1,3,5-trinitro-1,3,5-triazine in soils. *Soil Sci.*, 160:328–339.
55. Sembries, S. and Crawford, R.L., 1997, Production of *Clostridium bifermentans* spores as inoculum for bioremediation of nitroaromatic contaminants. *Appl. Environ. Microbiol.*, 63: 2100–2104.
56. Siciliano, S.D., Roy, R., and Greer, C.W., 2000, Reduction in denitrification activity in field soils exposed to long term contamination by 2,4,6-trinitrotoluene (TNT). *FEMS Microbiol. Ecol.*, 32:61–68.
57. Spanggord, R.J., Mortelmans, K.E., Griffin, A.F., and Simmon, V.E., 1982, Mutagenicity in *Salmonella typhimurium* and structure-activity relationships of waste water components emanating from the manufacture of trinitrotoluene. *Environ. Mutagen.*, 4:163–179.
58. Styles, J.A. and Cross, M.F., 1983, Activity of 2,4,6-TNT in an *in vitro* mammalian gene mutation assay. *Cancer Lett.*, 20:103–108.
59. Tadros, M.G., Crawford, A., Mateo-Sullivan, A., Zhang, C., and Hughes, J.B., 2000, Toxic effects of hydroxylamino intermediates from microbial transformation of trinitrotoluene and dinitrotoluenes on algae *Selenastrum capricornutum*. *Bull. Environ. Contam. Toxicol.*, 64:579–585.
60. Tan, E.L., Ho, C.H., Griest, W.H., and Tyndall, R.L., 1992, Mutagenicity of trinitrotoluene and its metabolites formed during composting. *J. Toxicol. Environ. Health*, 36:165–175.
61. Vaatanen, A.R., 1997, Spectrum of spontaneous and 2,4,6-trinitrotoluene (TNT)-induced mutations in *Salmonella typhimurium* strains with different nitroreductase and *o*-acetyltransferase activities. *Mutat. Res.*, 379:185–190.
62. Widrig, D.L., Boopathy, R., and Manning, J.F., 1997, Bioremediation of TNT-contaminated soil: A laboratory study. *Environ. Toxicol. Chem.*, 16:1141–1148.
63. Wikstrom, P., Andersson, A.C., Nygren, Y., Sjostrom, J., and Forsman, M., 2000, Influence of TNT transformation on microbial community structure in four different lake microcosms. *J. Appl. Microbiol.*, 89:302–308.
64. Won, W.D. and Disalvo, L.H.N.J., 1976, Toxicity and mutagenicity of 2,4,6-trinitrotoluene and its microbial metabolites. *Appl. Environ. Microbiol.*, 31:576–580.
65. Zhou, J.Z. and Tiedje, J.M., 1995, Gene transfer from a bacteria injected into an aquifer to an indigenous bacterium. *Mol. Ecol.*, 4:613–619.

CATABOLISM AND BIOTRANSFORMATIONS

ARGININE AND POLYAMINE METABOLISM

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1. INTRODUCTION

Arginine can serve many bacteria as a source of carbon, energy, and nitrogen, as a building unit of proteins, and as a precursor of polyamine synthesis^{14, 27}. It can also be an ammonia source for bacterial adaptation to acid environments^{13, 59, 101}. All of the major arginine catabolic pathways initiated by arginase, arginine deiminase (ADI), arginine succinyltransferase (AST), arginine decarboxylase (ADC), or arginine dehydrogenase (ADH), occur in bacteria¹⁴. Many bacteria have more than one route to utilize arginine depending on the physiological purpose. *Pseudomonas aeruginosa* PAO1 possesses four pathways of arginine metabolism (ADI, AST, ADC and ADH; Figure 1). The ADI and AST pathway genes were first established in strain PAO1^{28, 39}. Several ADC and ADH pathway genes have been recently identified and characterized in this strain^{65, 68}.

Polyamines are ubiquitous polycations necessary for optimal cell growth^{11, 14, 27} and serve as good carbon and nitrogen sources for *P. aeruginosa*^{34, 52}. Biosynthesis of putrescine, a precursor of spermidine synthesis, proceeds with the decarboxylation of either arginine or its precursor ornithine (Figure 2). Argmatinase and *N*-carbamoylputrescine amidohydrolase of the ADC pathway

perform putrescine biosynthesis in this strain⁶⁷. The presence of multiple pathways for arginine catabolism and the participation of some arginine catabolic enzymes in polyamine biosynthesis or catabolism constitute the complex metabolic networks of arginine and polyamines in *P. aeruginosa* PAO1.

This chapter illustrates the known networks of arginine and polyamine metabolism in *P. aeruginosa* PAO1 and review progress made over the last decade. The metabolic networks allow over 20 compounds to be utilized and represent elaborated metabolic systems developed by nutritionally versatile pseudomonads. Global and specific network regulation is also focused upon here. Global regulation controls, for example, cellular functions for adaptation to changes in oxygen tension or nutritional availability, while specific regulation accommodates several biological processes to achieve efficient utilization of the respective substrates.

2. ARGININE NETWORK

In *P. aeruginosa* PAO1, arginine biosynthesis proceeds from L-glutamate in eight enzymatic steps as in *E. coli* (Figure 1 and Table 1)^{14, 27}, except that *N*-acetylornithine acetyltransferase (encoded by *argJ*) also participates in *N*-acetylglutamate synthesis (Figure 1). The AST and ADI pathways consist of 5 and 3 enzymes respectively, and the ADC and ADH pathways include 12 enzymes (Figure 1). In addition, at least about 20 proteins are involved in transport and regulation. Thus, over 50 proteins are involved in the arginine network in strain PAO1.

2.1. Arginine Biosynthesis

All of the arginine biosynthetic genes (*argABCEFGHJ* and *carAB*) except for *argD*, which encodes *N*-acetylornithine aminotransferase, have been identified in *P. aeruginosa* PAO1^{40, 47, 88} (for gene products and functions, see reviews [14], [27], [28]). *N*²-succinylornithine aminotransferase (the *aruC* product) of the AST pathway is more similar (67% identity) to ArgD than to its counterpart AstC (61% identity) of *Escherichia coli*. AstC appears unable to perform an efficient anabolic function in vivo, as *argD* mutants of *E. coli* require arginine for growth³⁹. AruC can use *N*²-acetylornithine as a substrate in vitro as efficiently as *N*²-succinylornithine^{99, 105} and can complement the arginine-auxotrophic phenotype of an *E. coli argD* mutant³⁹, but *aruC* mutants of *P. aeruginosa* are prototrophic for arginine. This does not rule out a role for AruC in *N*²-acetylornithine synthesis but indicates that ArgD of *P. aeruginosa* PAO1 is less specific than the *E. coli* counterpart. The identities of all possible transaminases of this strain to *E. coli* ArgD are below 40%⁸⁸. Moreover,

Table 1. The arginine and polyamine catabolic genes of *P. aeruginosa* PAO1.

Gene (location) ^a	Enzyme (EC number)	Reaction catalyzed	References
Arginine catabolism			
<i>arcA</i> (5823)	Arginine deiminase (3.5.3.6)	L-arginine + H ₂ O → L-citrulline + NH ₄ ⁺	[28]
<i>arcB</i> (5824)	Ornithine carbamoyltransferase (2.1.3.3)	L-citrulline + Pi → ornithine + carbamoylphosphate	[28]
<i>arcC</i> (5825)	Carbamate kinase (2.7.2.2)	carbamoylphosphate + ADP → ATP + NH ₄ ⁺ + CO ₂	[28]
<i>aguA</i> (330)	Agmatine deiminase (3.5.3.12)	Agmatine + H ₂ O → N-carbamoylputrescine + NH ₄ ⁺	[67], [68]
<i>aguB</i> (331)	N-Carbamoylputrescine amidohydrolase (3.5.1.53)	N-carbamoylputrescine + 2 H ₂ O → putrescine + NH ₄ ⁺ + CO ₂	[67], [68]
<i>aruB</i> (983)	N ² -Succinylarginine dihydrolase (3.5.3.—)	N ² -succinylarginine + 2 H ₂ O → N ² -succinylornithine + 2 NH ₄ ⁺ + CO ₂	[39], [42], [99]
<i>aruC</i> (978)	N ² -Succinylornithine aminotransferase (2.6.1.13)	N ² -succinylornithine + 2-ketoglutarate → N-succinylglutamic semialdehyde + L-glutamate	[39], [42], [99]
<i>aruF</i> (980)	Arginine-ornithine succinyltransferase (AruA1 subunit) (2.3.1.109)	L-arginine + succinyl-CoA → N ² -succinylarginine + CoA	[39], [97], [99]
<i>aruG</i> (981)	Arginine-ornithine succinyltransferase (AruAII subunit) (2.3.1.109)	L-ornithine + succinyl-CoA → N ² -succinylornithine + CoA	
<i>aruD</i> (982)	N-Succinylglutamate semialdehyde dehydrogenase (1.5.1.12)	N-succinylglutamic semialdehyde + NAD ⁺ → N-succinylglutamate + NADH + H ⁺	[39], [42], [99]
<i>aruE</i> (985)	N-Succinylglutamate desuccinylase (3.5.1.—)	N-succinylglutamate → L-glutamate + succinate	[39], [42], [99]

<i>gbuA</i> (1547)	Guanidinobutyrase (3.5.3.7)	4-guanidinobutyrate + H ₂ O → 4-aminobutyrate + urea	[65], [108]
<i>gdhB</i> (1435)	NAD-dependent glutamate dehydrogenase (1.4.1.3)	L-glutamate + H ₂ O + NAD ⁺ → 2-ketoglutarate + NH ₄ ⁺ + NADH + H ⁺	[51]
<i>kauB</i> (5983)	Multifunctional aldehyde dehydrogenase (1.2.1.—)	4-guanidinobutyraldehyde + NAD ⁺ → 4-guanidinobutyrate + NADH + H ⁺	[88]
		4-aminobutyraldehyde + NAD ⁺ → 4-aminobutyrate + NADH + H ⁺	
		3-aminopropionaldehyde + NAD ⁺ → β-alanine + NADH + H ⁺	
Polyamine and 4-aminobutyrate catabolism			
<i>gabD</i> (300)	Succinic semialdehyde dehydrogenase (1.2.1.24)	succinic semialdehyde + NAD ⁺ + H ₂ O → succinate + NADH + H ⁺	[88]
<i>gabT</i> (302)	4-Aminobutyrate aminotransferase (2.6.1.19)	4-aminobutyrate + 2-ketoglutarate → succinic semialdehyde + L-glutamate	[88]
<i>spuC</i> (337)	Putrescine aminotransferase (2.6.1.29)	putrescine + pyruvate → 4-aminobutyraldehyde + alanine	[52]
<i>spdH</i> (4359)	Spermidine/spermine dehydrogenase (1.5.99.6)	spermidine + acceptor → 4-aminobutyraldehyde + 1,3-diaminopropane + reduced acceptor	Our unpublished data
		spermine + acceptor → spermidine + 3-aminopropanaldehyde + reduced acceptor	

^aGenome position (kb) defined by Stover *et al.*⁸⁸

other transaminase, such as 4-aminobutyrate aminotransferase¹⁰⁶, which has a broad substrate specificity, might execute the ArgD function in *P. aeruginosa*.

In *P. aeruginosa* PAO1, *N*-acetylglutamate synthase (*argA*) and glutamate acetyltransferase (*argJ*) catalyze *N*-acetylglutamate synthesis in the first reaction of arginine biosynthesis (Figure 1). ArgA essentially initiates arginine biosynthesis (*argA* mutants are arginine auxotrophs)²⁹, and ArgJ helps to minimize the amounts of acetyl-CoA consumed by ArgA, by recycling the acetyl group in the pathway. *E. coli* has only ArgA²⁷, whereas *Bacillus subtilis* has only ArgJ that probably assumes the anaplerotic function of *N*-acetylglutamate synthase⁷. *P. aeruginosa* PAO1 controls cellular arginine pools through feedback inhibition of the first two enzymes and by modulating the transcription of *argF* (for anabolic ornithine carbamoyltransferase) and *carAB* (carbamoylphosphate synthetase) (see specific regulation section). This is in contrast to *E. coli* and *B. subtilis* where the entire arginine biosynthetic regulon is controlled at the transcription level^{7, 14, 27}.

2.2. Arginine Catabolism

2.2.1. ADI Pathway

The *arcDABC* operon encodes an arginine–ornithine antiport protein (*arcD*) and the ADI pathway enzymes^{9, 28, 53}, ADI (*arcA*), catabolic ornithine carbamoyltransferase (*arcB*), and carbamate kinase (*arcC*) (Figure 1 and Table 1). The primary role of this pathway is to supply ATP to the “strict aerobic” *P. aeruginosa* PAO1 for driving motility and nutrient uptake as well as for slow growth under anaerobic conditions²⁸. This pathway is induced by oxygen limitation and generates copious amounts of ammonia when sufficient arginine is present in the medium. In fact, the detection of ammonia production using a pH-indicator has identified the pathway as a taxonomic marker in bacterial classification^{28, 80}. This pathway contributes to the survival of PAO1 cells in acidic conditions by neutralizing the acidity via the production of ammonia⁵⁹. It is also important in the pH-control of the fermentative growth of cultured lactic bacteria that produce potentially lethal amounts of acids^{17, 19, 59}.

Ornithine (yielded in the ArcB reaction) is another pathway product that is not further metabolized under anaerobic conditions. It is used to drive arginine uptake. The integral membrane protein ArcD mediates a stoichiometric and energy-independent exchange between ornithine produced in the cytosol and arginine in the medium^{9, 102}. This transport mechanism thus allows energy-deficient anaerobic cells to take up arginine without using energy.

The catabolic ArcB enzyme shares 52% similarity with the anabolic ArgF enzyme, but they have different subunit structures and perform unidirectional reactions in vivo^{6, 40, 58, 93}. ArcB is a dodecamer consisting of four trimers of equal subunits, whereas ArgF is a trimer of identical subunits.

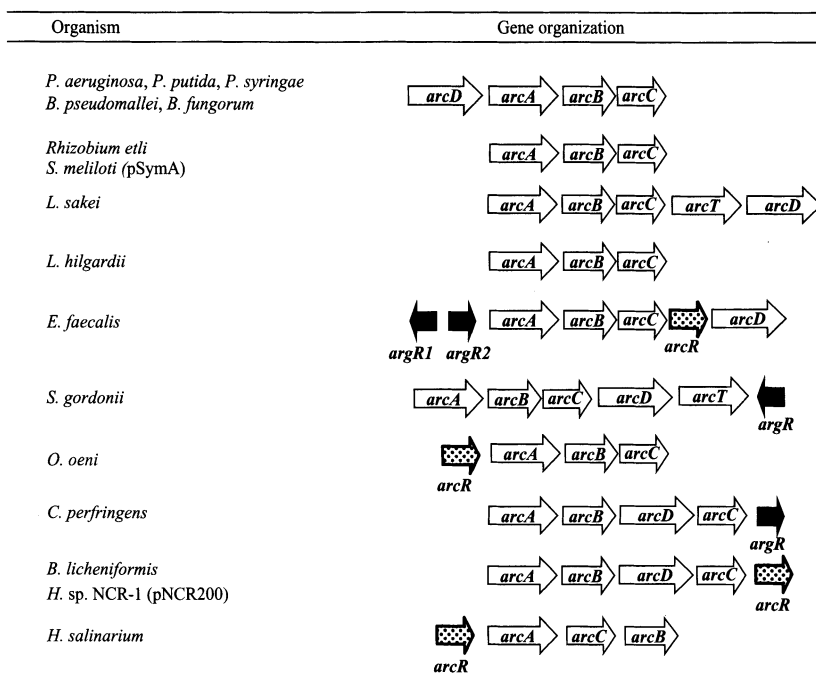


Figure 3. Organization of *arc* genes in various species. Sequence data are available at KEGG (<http://mbgd.genomne.ad.jp>) and NCBI (<http://www.ncbi.nlm.nih.gov>). The *arcT* genes of *Lactobacillus sakei* and *Streptococcus gordonii* encode a putative transaminase and Xaa-His peptidase, respectively^{19, 110}. The regulatory gene *argR* encodes the ArgR (*E. coli*)/AhrC (*B. subtilis*)-type arginine repressor and *arcR* is a gene for Crp/Fnr-type anaerobic regulator.

ArcB cannot catalyze the thermodynamically favored formation of citrulline and phosphate from ornithine and carbamoylphosphate *in vivo*, due to low affinity and high cooperativity toward carbamoylphosphate^{6, 58}. Glutamate at position 105 (Glu105) as well as at the N- and C-terminals are responsible for the carbamoylphosphate cooperativity^{6, 72, 73, 94}. Glu105 is buried within a hydrophobic core of the catalytic site for carbamoylphosphate and makes a salt bridge with nearby Arg107 (a residue for carbamoylphosphate binding and cooperativity) to compensate for its thermodynamically unfavorable negative charge¹⁰⁴. Amino acid substitution of E105 by a neutral or basic residue (alanine, glycine, or lysine) abolishes carbamoylphosphate cooperativity and confers an anabolic function upon the enzyme^{6, 93, 97}. The C-terminal Ile335 forms a salt bridge with the N-terminal His5 on the same monomer or with the N-terminal Lys45 of another monomer^{93, 104}. Deletions or substitutions of the C-terminal residue abolish or diminish cooperativity toward carbamoylphosphate⁷³. Similar replacement of the N-terminal regions 1–26 or 1–42 with the

corresponding *E. coli* ArgF sequence reduces the cooperativity⁷². These structures alone however do not significantly influence dodecamer formation and the trimer itself displays homotropic cooperativity. A dodecameric structure is therefore not necessary for cooperativity, but highly organized oligomerization appears to enhance the intrinsic allosteric property and would ultimately be responsible for the inactivity of ArcB in the anabolic function^{63, 72, 97}.

The *arc* operon has been identified and characterized in a variety of eubacteria and in archaea (Figure 3). Orthologues also occur in *Staphylococcus aureus*, *Lactococcus lactis*, and *Mycoplasma penetrans* as well as in α -proteobacteria (*Sinorhizobium meliloti*), and other γ -proteobacteria (*P. fluorescens*, *Salmonella typhi*, and *S. typhimurium*) (<http://www.ncbi.nlm.nih.gov>). The gene order of *arcDABC* is perfectly conserved in *Burkholderia pseudomallei* and *Burkholderia fungorum*, as well as in *Pseudomonas putida* and *Pseudomonas syringae*, but not in *Rhizobium etli*¹⁶ (Figure 3). In contrast, the *arc* genes of Gram-positive bacteria are divergent in terms of gene organization and a putative transaminase gene (*arcT*) and/or regulatory genes (*arcR* and *argR*) are often located within or adjacent to the gene cluster (Figure 3). Functions of the regulatory genes are discussed in a later section. Plasmids pSymB and pNCR200 encode the *arcABC* genes in *S. meliloti* and *Halobacterium* spp., respectively. The fact that these plasmid-borne *arc* genes have the same order as the chromosomal *arc* genes of *R. etli* and *Halobacterium salinarium* (formerly *Halobacterium halobium*) (Figure 3) makes their horizontal transmission among these and related species plausible.

2.2.2. AST Pathway

The AST pathway is the major catabolic route of arginine and ornithine as a carbon source in *P. aeruginosa* PAO1^{39, 42, 98}. This pathway starts with the succinyl-CoA-dependent succinylation of arginine by succinyltransferase (encoded by *aruFG* at the *aruA* locus)^{39, 96}. *N*²-succinylarginine dihydrolase (*aruB*) converts *N*²-succinylarginine into *N*²-succinylornithine, and this common intermediate of arginine and ornithine catabolism then leads to glutamate and succinate in three enzymatic steps (*aruCDE*) (Figure 1). Catabolic (NAD⁺-dependent) glutamate dehydrogenase (*gdhB*)⁵¹ converts the glutamate to 2-ketoglutarate, which is metabolized through the TCA cycle and regenerates succinyl-CoA for re-initiation of the pathway (Figure 1). Thus, when arginine is used as a carbon and energy source via the AST pathway, the carbon skeleton is essentially degraded in the TCA cycle.

AruA is a heterotetramer composed of two homologous subunits (AruAI and AruAII encoded by *aruF* and *aruG*, respectively) (Figure 4), which succinylates arginine as well as ornithine with succinyl-CoA⁹⁶. *P. aeruginosa* PAO1 can thereby also degrade ornithine through this pathway, although growth on ornithine is significantly slower than that on arginine^{96, 98}. Such

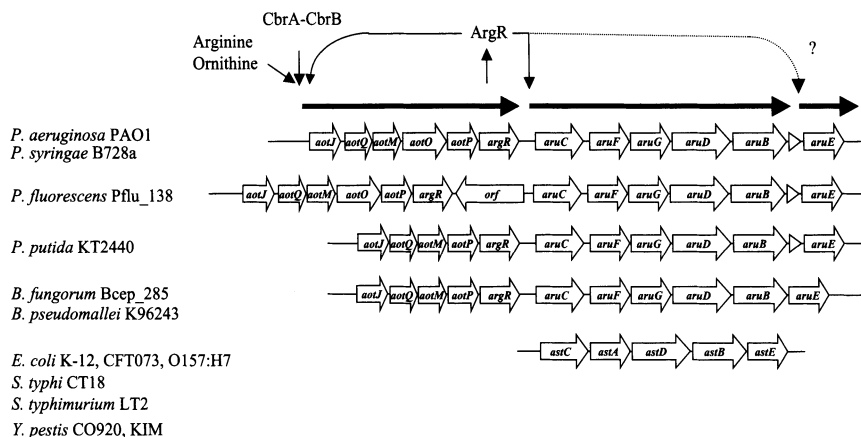


Figure 4. Structures and regulation of the *aot-argR* and *aru* operons. Sequence data are available in the databases indicated in the legend to Figure 3. Functions of each gene are listed in Tables 1 and 2.

slow growth on ornithine can be explained in part by the cooperativity of the enzyme toward ornithine. The ornithine concentration at half-maximal velocity can be as high as 25 mM, whereas the reaction with arginine proceeds in a hyperbolic curve and the K_m value of the enzyme for the substrate is 0.5 mM⁹⁶. Cells containing mutant AruA with reduced ornithine cooperativity grow faster on ornithine, but still more slowly than on arginine⁹⁶. Inefficient ornithine uptake and weak induction of the pathway enzymes by ornithine^{71, 98} also account for the slow growth on ornithine.

The AST enzymes have also been identified in *Aeromonas formicans*, *Klebsiella aerogenes*, *Escherichia coli*, *Burkholderia cepacia* (formally *Pseudomonas cepacia*), and in many other *Pseudomonas* strains^{14, 85, 95, 99}. Genome sequencing (<http://mbgd.genome.ad.jp>; <http://www.ncbi.nlm.nih.gov>) has identified the *aru* operon in other *Pseudomonas* strains (*P. putida*, *P. fluorescens*, and *P. syringae*), *Burkholderia* (*B. fungorum* and *B. pseudomallei*), enteric bacteria (*Escherichia coli*, *Salmonella typhi*, *Salmonella typhimurium*, and *Yersinia pestis*), *Vibrio* (*V. cholerae*, *V. parvulus*), *Caulobacter crescentus*, and in *Shewanella oneidensis*. No *aru/ast* homologues have yet been found in Gram-positive bacterial genomes. All *Pseudomonas* and *Burkholderia* strains examined so far have the same gene order of *aruCFGBDE*. This order is also conserved in enteric bacteria (where *aru* is termed as *ast* or *cst*), except that a single *astA* gene specifies the succinyltransferase^{22, 85} (Figure 4).

The AST pathway supplies cells with carbon and nitrogen sources or is only a nitrogen source depending on the strain. It is a major pathway of *P. aeruginosa* PAO1 for aerobic arginine utilization as carbon and nitrogen

sources^{39, 42, 98}. An *aru*-null mutation almost abolishes the arginine utilization by this strain as a carbon, but not as a nitrogen source, though it significantly affects growth with arginine supplied as the nitrogen source (our unpublished data). In PAO1 cells other pathways can supply nitrogen from arginine to support slow growth when the AST pathway is blocked.

2.2.3. ADH Pathway

The ADH pathway enzymes, L-arginine dehydrogenase (=oxidase), 2-ketoarginine decarboxylase, 4-guanidinobutyraldehyde dehydrogenase, and guanidinobutyrase, have been identified in *P. putida*^{12, 61, 100}. The first enzyme oxidatively deaminates L-arginine to produce 2-ketoarginine, which is subsequently converted by the following three enzymes to 4-aminobutyrate with the concomitant formation of urea and CO₂ (Figure 1, Table 1). *P. putida* also has the AST pathway (see above). The ADH and AST pathways operate simultaneously and equally contribute to arginine utilization by this bacterium⁹⁵. *P. aeruginosa* PAO1 does not metabolize L-arginine by this pathway, because of the apparent absence of L-arginine dehydrogenase, but it can utilize all compounds below 2-ketoarginine as carbon and nitrogen sources, as it has a complete set of the enzymes downstream of 2-ketoarginine decarboxylase⁴¹. The presence of a D-arginine-inducible D-arginine dehydrogenase, instead of L-arginine dehydrogenase, appears to enable this strain to utilize D-arginine via this pathway^{28, 41}. An *aruA* null mutant of strain PAO1 slowly utilizes D-arginine as well as L-arginine as a carbon source (our unpublished data), indicating that in the wild type, the D-enantiomer may be converted to the L-enantiomer by arginine racemase⁴¹ and channeled into the AST, as proposed for D-arginine metabolism by *P. putida*⁹⁵. An *aruA gbuA* double mutant of strain PAO1 is unable to utilize both L- and D-arginine as a carbon source but still able to do as a nitrogen source (our unpublished data). The ADH pathway therefore appears to be an auxiliary route of arginine catabolism as the carbon source and ADI and/or the ADC pathway may generate the NH₄⁺ involved.

Two ADH pathway genes, *kauB* (for 4-guanidinobutyraldehyde dehydrogenase) and *gbuA* (for guanidinobutyrase), have been identified in *P. aeruginosa* PAO1 (Table 1). KauB is a multifunctional aldehyde dehydrogenase that also participates in the catabolic conversion of 4-aminobutyraldehyde to 4-aminobutyrate and of 3-aminopropanaldehyde to β -alanine in polyamine metabolism⁴¹ (also see Section 3). The subunit structure and kinetic properties of this enzyme (deduced Mr of 53 kDa) remain unknown. GbuA is a homodimer of 140 kDa that is highly specific for 4-guanidinobutyrate^{65, 108}. The *gbuA* locus is separate from the *kauB* locus, and in their flanking regions the D-arginine dehydrogenase and the 2-ketoarginine decarboxylase genes are not present. Thus, the ADH pathway genes are scattered across at least three different loci.

2.2.4. ADC Pathway

P. aeruginosa PAO1 possesses an arginine-inducible ADC⁶⁰. Agmatine, the decarboxylation product of arginine, is a carbon and nitrogen source for *P. aeruginosa* PAO1. Agmatine is catabolically converted into putrescine, 2 NH₄⁺, and CO₂ in two successive reactions catalyzed by agmatine deiminase (*aguA*) and *N*-carbamoylputrescine amidohydrolase (*aguB*) in this strain^{30, 68} (Figure 1 and Table 1). AguA is a homodimer of 43 kDa subunits with a novel sequence of C–N hydrolases that is highly specific for agmatine⁶⁷. AguB is a homohexamer of 33 kDa subunits that resemble the β -alanine synthetase/nitrilase family enzymes of C–N hydrolases and displays high specificity for *N*-carbamoylputrescine⁶⁷. The ADC pathway appears not to contribute to arginine utilization, since an *aruA gbuA* mutant cannot use arginine as a carbon source, as mentioned above. The *aguBA* operon (in the gene order given) is induced to high levels by exogenous agmatine, to a lesser extent by *N*-carbamoylputrescine, and marginally by arginine^{60, 68}. The ADC levels induced by arginine probably cannot yield agmatine in concentrations sufficient for *aguBA* induction.

Some *Pseudomonas* strains (e.g., *Pseudomonas alcaligenes*) have this pathway as a unique catabolic route of arginine utilization as a nitrogen source¹⁴. Whether this pathway can serve for *P. aeruginosa* PAO1 as a nitrogen route of arginine metabolism remains to be explored.

3. POLYAMINE NETWORK AND ITS LINK TO THE ARGININE NETWORK

Putrescine and spermidine are major polyamines in bacteria and spermine also occurs in eukaryotic cells^{11, 15}. The biosynthesis of putrescine proceeds from either arginine or its precursor ornithine in *P. aeruginosa* PAO1. This section outlines the biosynthetic and metabolic networks of polyamines in which KauB and AguAB are involved as catabolic and biosynthetic enzymes, respectively.

3.1. Polyamine Biosynthesis

P. aeruginosa PAO1 can generate putrescine directly from ornithine by ornithine decarboxylase (ODC, the *speC* product) or indirectly from arginine via biosynthetic ADC encoded by *speA*⁶⁷ (Figure 2). Subsequent transfer of the aminopropyl group from decarboxylated S-adenosylmethionine to putrescine by spermidine synthase (*speE*) results in spermidine synthesis. Either route alone can supply putrescine in amounts sufficient to support normal cell growth. SpeC decarboxylases of strain PAO1 (deduced Mr of 43,559) has no

sequence similarity with *E. coli* SpeC (Mr 79,414)¹⁰ but resembles the counterparts of Archaea, for example, *Methanosarcina mazei* (60% identity), and *Saccharomyces cerevisiae* (30% identity). Anabolic conversion of agmatine to putrescine can proceed directly via agmatinase (*speB*) (e.g., in *E. coli*) or successively via agmatine deiminase (*aguA*) and *N*-carbamoylputrescine amidohydrolase (*aguB*) (e.g., in plants)^{11, 27}. *P. aeruginosa* PAO1 lacks SpeB and recruits AguAB for putrescine biosynthesis⁶⁷ (Figure 2). Thus, AguAB performs both putrescine biosynthesis and agmatine metabolism in *P. aeruginosa* PAO1. The AguAB sequences led to the identification of cDNA clones for AguB (62% identity)⁷⁹ and a putative AguA (56% identity) in *Arabidopsis thaliana*, tomato, and rice; AguAB also have counterparts in many *Pseudomonas* and other bacterial species^{67, 68}. The absence of a *speB* orthologue in these organisms implies that *aguAB* orthologues participate in putrescine biosynthesis and that some of the bacterial orthologues may also be involved in agmatine metabolism like the *P. aeruginosa* enzymes.

Constitutive levels of AguAB are below 10% of the induced levels, but can complete putrescine synthesis in amounts sufficient for normal growth even when *speC* is blocked⁶⁷. Exogenous arginine coordinately doubles ADC and AguAB synthesis and inversely represses SpeC formation by 75%, whereas putrescine prevents ADC and ODC synthesis by 50–70%⁶⁷. Ornithine has no apparent regulatory property. The inverse regulation of the ADC and ODC by arginine can avoid futile ODC synthesis when ornithine levels are low as a consequence of feedback inhibition of the biosynthetic enzymes by exogenous arginine and allows efficient putrescine synthesis from arginine. The regulation of *speDE* remains to be studied in *P. aeruginosa* PAO1.

3.2. Polyamine Catabolism

Putrescine that is formed internally from agmatine via AguAB or supplied exogenously is catabolized into 4-aminobutyraldehyde by putrescine aminotransferase (encoded by *spuC*). SpuC belongs to the class III of pyridoxal-phosphate-dependent aminotransferases⁵². It prefers pyruvate as an amino acceptor and is only weakly similar (27% identity) to the *E. coli* counterpart (*ygiG*), which uses 2-ketoglutarate as an amino acceptor⁸⁴. The *spuC* gene is integrated in the *spuABCDEFGH* operon that encodes putative enzymes (*spuAB*) of unknown function and the spermidine–spermine transport proteins (*spuDEFGH*) that constitute a periplasmic binding protein-dependent transport system homologous to that of *E. coli* Pot polyamine uptake⁵². The inactivation of *spuC* abolishes the utilization of putrescine, but not that of spermidine and spermine, both of which can be metabolized via 3-aminopropanaldehyde to β -alanine (Figure 2; see below). By contrast, any *spuDEFGH* mutation results in defective spermidine and spermine utilization

with no effect on putrescine utilization. Thus, the SpuDEFGH system is the specific transport pathway of spermidine and spermine and the PotABCD system (www.pseudomonas.com) mediates putrescine transport as in *E. coli*^{27,52}.

Many bacteria including *P. aeruginosa* utilize spermidine and spermine as carbon and/or nitrogen sources^{34, 89}, but bacterial polyamine catabolic enzymes have not been studied in detail. Spermidine dehydrogenase (Table 1) was first identified and purified in *Serratia marcescens* as a possible catabolic enzyme of spermidine⁸⁹. This enzyme is a spermidine-inducible flavoprotein of 76 kDa associated with the cytoplasmic membranes. Thereafter, similar enzymes were identified in *Citrobacter freundii* and in other bacteria including *P. aeruginosa*^{34–36}. The *C. freundii* enzyme (63 kDa) is also associated with the cytoplasmic membrane in cells cultured with spermidine. It contains pyrroloquinoline quinone instead of FAD as the prosthetic group³⁵. The *P. aeruginosa* enzyme shares similar properties with the *C. freundii* enzyme but is constitutive³⁴. We identified PA3713 (<http://www.pseudomonas.com>) as the spermidine dehydrogenase gene (*spdH*) of *P. aeruginosa* PAO1 using the determined N-terminal sequence. SpdH is similarly active against spermidine and spermine. It cleaves spermidine to 4-aminobutyraldehyde and 1,3-diaminopropane and spermine to spermidine and 3-aminopropanaldehyde, thus yielding 4-aminobutyraldehyde, 1,3-diaminopropane, and 3-aminopropanaldehyde from spermine as the final products (Figure 2). A knockout mutant of *spdH* however can still grow on both spermidine and spermine as carbon and nitrogen sources and appears to have a polyamine-inducible degradation enzyme for the polyamines. Whether either or both of these enzymes are responsible for polyamine utilization by *P. aeruginosa* PAO1 is presently unknown. Identification and inactivation of the gene for the inducible enzyme in the wild type and the *spdH* background should resolve this question.

The 4-aminobutyraldehyde that results from putrescine by SpuC is oxidized to 4-aminobutyrate (GABA) by the NAD⁺-dependent KauB aldehyde dehydrogenase, which also catalyzes the oxidative conversion of 4-guanidinobutyraldehyde to 4-guanidinobutyrate in the ADH pathway (see above; Figure 2). We found that *kauB* mutants cannot use 1,3-diaminopropane, spermidine, and spermine, in addition to putrescine and 2-ketoarginine⁴¹; (our unpublished data). Such growth phenotypes of the *kauB* mutants support the notion that both spermidine and spermine are metabolized via 3-aminopropanaldehyde and 4-aminobutyraldehyde as intermediates (Figure 2). Beta-alanine, the oxidation product derived from 3-propanaldehyde by KauB, is then converted to malonic semialdehyde by β -alanine aminotransferase and subsequently to acetyl-coenzyme A (acetyl-CoA) by malonic semialdehyde dehydrogenase, before condensation into citrate via citrate synthase (*gltA*)¹⁸. The possible genes for β -alanine aminotransferase (PA0132) and malonic semialdehyde dehydrogenase (PA0130) have been assigned according to

Table 2. The transport and regulatory genes in the arginine and polyamine networks of *P. aeruginosa* PAO1.

Gene (location) ^a	Property or function	References
<i>Transport genes</i>		
Arginine and ornithine transport		
<i>arcD</i> (5822)	Ornithine-arginine antiporter	[9], [28], [102]
<i>aotJ</i> (972)	Periplasmic arginine/ornithine-binding protein	[71]
<i>aotQ</i> (973)	Membrane transport protein	[71]
<i>aotM</i> (974)	Membrane transport protein	[71]
<i>aotP</i> (976)	ATP-binding cassette (ABC) transport protein	[71]
Polyamine transport		
<i>spuD</i> (339)	Periplasmic spermidine/spermine-binding protein	[52]
<i>spuE</i> (341)	Periplasmic spermidine/spermine-binding protein	[52]
<i>spuF</i> (342)	Membrane transport protein	[52]
<i>spuG</i> (343)	Membrane transport protein	[52]
<i>spuH</i> (344)	ABC transport protein	[52]
<i>potA</i> (4041)	Possible periplasmic putrescine-binding protein	[88]
<i>potB</i> (4042)	Membrane transport protein	[88]
<i>potC</i> (4043)	Membrane transport protein	[88]
<i>potD</i> (4044)	ABC transport protein	[88]
4-Aminobutyrate transport		
<i>gabP</i> (146)	4-Aminobutyrate permease	[88]
<i>Regulatory genes</i>		
<i>argR</i> (977)	ArcA/XylS family, positive and negative regulator of arginine network	[76], [77]
<i>aguR</i> (331)	TetR family, repressor of the <i>aguBA</i> operon	[68]
<i>anr</i> (1681)	Crp/Fnr family, anaerobic regulator	[23], [28]
<i>cbrA</i> (5035)	Sensor-histidine kinase	[70]
<i>cbrB</i> (5037)	Response regulator (NtrC-type)	[70]
<i>gbuR</i> (154)	LysR family protein, expression of <i>gbuA</i> induced by 4-guanidinobutyrate	[65]

^aGenome position (kb) defined by Stover *et al.*⁸⁸

similarities to the β -alanine aminotransferase of *P. putida* (87% similarity) and the methylmalonic semialdehyde dehydrogenase of *P. aeruginosa* PAO1 (67% similarity) (www.pseudomonas.com), respectively.

4. THE GABA PATHWAY, CONFLUENCE OF THE NETWORKS

At the confluence of the ADH and ADC-putrescine catabolic pathways, 4-aminobutyrate is channeled into the TCA cycle via 4-aminobutyrate aminotransferase (*gabT*) and succinic semialdehyde dehydrogenase (*gabD*) (Figure 1). The *gabDT* genes of *E. coli* and *B. subtilis* form an operon together with *gabP* (GABA permease) and the *gabC* (*E. coli*) or *gabR* (*B. subtilis*) regulatory gene^{5, 8, 69, 86}. The *gabDT* genes of *P. aeruginosa* PAO1 (Table 2) appear to constitute an operon, but the *gabP* gene (77% similar to *E. coli gabP*) is located at a different locus and a regulatory gene elsewhere has not been identified (www.pseudomonas.com).

5. SPECIFIC REGULATIONS

ArgR is an AraC/XylS family protein^{21, 24, 77, 90} that positively controls the AST and ADI pathways and negatively controls the *argF* and *carAB* genes in response to exogenous arginine. The ADC, ADH, and polyamine catabolic pathways comprise distinct regulatory units that are regulated by different pathway intermediates. This section describes the regulatory mechanism underlying the inverse regulation by ArgR and the regulation of each pathway unit that enables consecutive expression of the corresponding pathways.

5.1. Regulation by ArgR

The *aotJQMOP-argR* operon immediately upstream of the *aru* operon^{71, 76} (Figure 4) encodes a periplasmic binding-protein dependent arginine–ornithine transport system (AotJQMP) and the ArgR regulatory protein (Table 2). This operon structure is conserved among *Pseudomonas* and *Burkholderia* species, except that *P. aeruginosa* as well as *P. fluorescens* have *aotO* without a transport function between *aotM* and *aotP*⁷¹ and *P. fluorescens* has the additional insertion of a hypothetical gene between *argR* and *aruC* (Figure 4). Arginine controls cellular amounts of ArgR by modulating the transcription of the operon. The 5'-flanking region of *aotJ* carries two promoters (P1 and P2) for the operon. In the absence of arginine (or ornithine), transcription from the upstream promoter P1 occurs at low levels. When the inducer is present, transcription from P1 ceases and P2 actively expresses the

operon at high levels instead. This promoter switch is accomplished by ArgR binding to the 21-bp tandem repeat (ArgR box) that is located adjacent to the -35 region of P2 and overlaps with the -10 region of P1⁷¹. ArgR bound to the box interferes with the access of RNA polymerase to P1 and recruits the enzyme to P2 to stimulate transcription from this promoter. Through binding to the cognate ligand at the C-terminal domain, the AraC/XylS family of proteins (e.g., AraC, MelR, XylS, etc.) modifies the binding affinity to operators or become an active dimer^{21, 24}. ArgR can form a dimer and bind to the ArgR box, independently of arginine^{70, 77}. Whether ArgR has binding affinity to arginine is unknown. Both arginine and ArgR are essential, but not sufficient for P2 activation. Transcription from P2 also requires the functions of the CbrA-CbrB two-component regulatory proteins⁷⁰. A possible role of arginine in the regulation of P2 is considered in conjunction with that of the CbrA-CbrB pair in the next section.

ArgR also activates the expression of the *aru* and *arc* operons and the *oprD* and *gdhB* genes (Table 2). The *aruC* promoter has two ArgR boxes (Figure 5), perhaps facilitating the recruitment of RNA polymerase to the promoter for efficient expression of the major catabolic pathway. In contrast, anaerobic expression of the *arc* operon is essentially activated by the Anr regulatory protein (see below), as arginine does not induce the *arc* operon in aerated cells or in Anr-negative cells²⁸. However, ArgR stimulates Anr-dependent expression when it binds to the ArgR box adjacent to the Anr-binding site⁵⁰ (Figure 5). ArgR appears to promote the initiation of *arc* transcription through physical contact with Anr⁵⁰. This view implies that ArgR can cooperate with other regulators, a property unique to ArgR within this family. OprD is an outer membrane porin that constitutes a diffusion channel for basic amino acids and peptides as well as the antibiotic imipenem⁷⁴. OprD synthesis is enhanced by arginine, alanine, glutamate, or histidine, and an *argR* mutation specifically abolishes arginine-dependent synthesis. The ArgR box on the *oprD* promoter is separated by 22 bp from the -35 sequence. This space might have a binding sequence for a factor involved in regulation by other amino acids or for another factor like in the *arcD* promoter (Figure 5). The *gdhB* gene is also activated during growth on arginine, as a consequence of ArgR binding to the ArgR-box adjacent to the promoter⁵¹ (Figure 5). OprD porin could facilitate arginine diffusion across the outer membrane and GdhB may help efficient channeling of glutamate, an AST pathway product, into the TCA cycle via 2-ketoglutarate^{51, 74}.

On the contrary, ArgR binding to the ArgR boxes within the -10 and -35 regions of the *argF* and *carA* promoters represses expression of these arginine biosynthetic genes⁷⁷. In addition to feedback inhibition of the first two arginine biosynthetic enzymes, arginine-dependent repression of the *argF* and *carAB* biosynthetic genes ensures a tight block of arginine biosynthesis

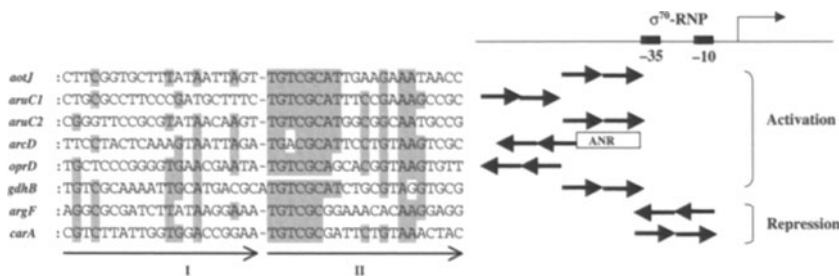


Figure 5. Sequence alignment of ArgR-binding sites and their locations in arginine-responsive promoters. Arrows below alignment indicate first and second halves (I and II) of the binding sequences. σ^{70} -RNP, σ^{70} -dependent RNA-polymerase holoenzyme; -10 and -35, promoter sites for σ^{70} -RNP. Arrows facing left indicate binding sequences on complementary strands. This figure was taken from Lu and Abdelal⁵¹ with permission and is slightly modified.

and prevents the futile consumption of ATP used for carbamoylphosphate synthesis²⁷ when exogenous arginine is available. ArgR thus coordinates the arginine network by functioning as either a positive or a negative regulator in response to arginine availability.

In enteric bacteria and *B. subtilis*, ArgR and AhrC repressors, respectively, which are homologous to each other but have no structural relatedness to *P. aeruginosa* ArgR, negatively control the arginine biosynthetic *arg* regulon in response to exogenous arginine^{7, 27, 45}. ArgR of *E. coli* and *S. typhimurium* can enhance the arginine-dependent expression of the *ast* operons^{44, 49}, and AhrC of *Bacillus licheniformis* mediates arginine-dependent expression of the catabolic *arcABDC* operon⁵⁷. Thus, the arginine regulators that belong to distinct protein families control negatively arginine biosynthesis and positively arginine catabolism through very similar mechanisms.

5.2. Sequential Regulation of the ADC and ADH Pathway

Syntheses of the ADC and ADH pathway enzymes are differently controlled. AguR, a TetR-type repressor encoded by the *aguR* gene immediately upstream of *aguBA*, controls inducible expression of the *aguBA* operon by agmatine or *N*-carbamoylputrescine^{30, 68}. In the absence of the substrate, the repressor binds to a palindromic sequence (TCCGATTTTTATCGGA) between the -10 and -35 regions to prevent the RNA polymerase holoenzyme from accessing the promoter. Agmatine as well as *N*-carbamoylputrescine antagonize the binding activity of AguR to induce *aguBA* expression⁶⁸. Agmatine, putrescine, spermidine, and spermine actively express the *spuABCDEFGH* operon⁵². Since the induction effect of agmatine is abolished in an *aguA* mutant⁵², agmatine exerts its induction effect via one of

its metabolites, probably putrescine. Such polyamine-dependent expression is consistent with the operon functions of *SpuC* transaminase for putrescine metabolism (Figure 2) and *SpuDEFGH* transport proteins for spermine and spermine uptake (see above). PAO1 cells cultured with a polyamine produce a protein that can bind to the *spuA* promoter⁵². Characterization of this putative regulatory protein and the corresponding gene would shed light on how the *spu* operon is regulated by polyamines.

The ADH pathway of *P. putida* P2 comprises several regulatory units, namely L-arginine transport system, L-arginine dehydrogenase (=oxidase), 2-ketoarginine decarboxylase, and 4-guanidinobutyraldehyde dehydrogenase and 4-guanidinobutyrate amidinohydrolase (=guanidinobutyrase)¹⁰⁰. The *P. aeruginosa* PAO1 pathway enzymes seem to be similarly regulated, except that the synthesis of D-arginine dehydrogenase is induced by D-arginine. In this strain 2-ketoarginine induces 4-guanidinobutyraldehyde dehydrogenase (*KauB*) synthesis and 4-guanidinobutyrate expresses guanidinobutyrase synthesis⁴¹. The regulation of 2-ketoarginine decarboxylase synthesis has not been studied in strain PAO1 and the decarboxylase and dehydrogenase genes have not been identified in either strain, leaving the regulatory mechanisms of these genes obscure. Putrescine and 4-guanidinobutyraldehyde as well as 2-ketoarginine, can provoke *KauB* formation^{41, 100}. The regulatory gene for *kauB* has not been identified, but the induction response of this gene supports the notion that 4-guanidinobutyraldehyde and 4-aminobutyraldehyde are probable inducers of this gene. The expression of *gbuA* encoding the last pathway enzyme is induced by 4-guanidinobutyrate through the function of *GbuR*, a regulatory protein of the LysR family encoded by the divergent *gbuR* gene upstream of *gbuA*⁶⁵.

The ADC-putrescine and ADH pathways join at GABA. The GABA catabolic enzymes (*GabT* and *GabD*) are induced by the upstream intermediates, 4-guanidinobutyrate, putrescine, and spermidine (in that order), as well as by GABA, in both *P. aeruginosa* and *P. putida*^{95, 106}. The *gab* operons of *E. coli* and *B. subtilis* are also inducible by GABA, but through a different mechanism. The *GabC* repressor negatively controls the *E. coli* operon, while the *GabR* protein of *B. subtilis* is a transcription activator with an autoregulatory property^{8, 86}. Since genes similar to *gabC* or *gabR* have not been found around the *gabDT* operon of *P. aeruginosa*, the regulatory mechanism remains unknown.

6. GLOBAL REGULATION

Two global regulatory systems, the anaerobic regulator *Anr* and the *CbrA-CbrB* two-component regulatory proteins, control the anaerobic and

aerobic metabolism of arginine, respectively. This section discusses the regulatory mechanisms of the two regulatory systems and considers the physiological significance of global controls.

6.1. Anr

The anaerobic regulator Anr, a Crp/Fnr-type transcription activator, plays the major role in the regulation of the anaerobic functions in *P. aeruginosa* PAO1, including the nitrate respiration–denitrification pathway^{2, 3, 31, 43, 82, 103, 107, 109}, heme synthesis^{46, 83}, cyanide synthesis^{48, 78} (also see Chapter 27, Volume 3), and the ADI pathway^{23, 28}. The *arcDABC* operon is expressed as a polycistronic mRNA^{23, 25}. The Anr-binding site (TTGACGTGGATCAG) centered at –41.5 bp from the *arcD* transcription initiation site is responsible for the anaerobic *arc* expression²³. This sequence resembles the consensus Fnr box of *E. coli* (TTGAT . . . ATCAA)⁵⁴. The ArgR box located at –73.5 bp, directly upstream of the Anr box, accounts for auxiliary induction of the *arc* operon by ArgR, as described above.

In Gram-positive bacteria, the ArcR protein encoded by the *arcR* gene, which is often located within or adjacent to the *arc* operon (Figure 3), mediates anaerobic expression of the *arc* operon. ArcR proteins also belong to the Crp/Fnr family of proteins^{4, 56, 75, 91} but share little sequence homology (<20% identity) with Anr. The ArcR-binding region between –33 and –65 bp from the transcription initiation point of the *B. licheniformis arcA* gene contains a 21-bp sequence similar to the consensus Crp-binding sequence⁵⁶. In addition to anaerobiosis, Gram-positive bacteria require arginine to express the *arc* operon^{4, 19, 57, 110}. The binding site for an ArgR/AhrC-type arginine regulator on the *B. licheniformis arcA* promoter is situated immediately upstream of the ArcR-binding motif^{56, 57}. This promoter–operator topology is analogous to that in the *arcD* promoter of *P. aeruginosa* PAO1 (Figure 5), implying that the transcription mechanism of the *arc* operon is similar in *P. aeruginosa* and *B. licheniformis*. Expression of the *arc* operons of some Gram-positive bacteria is subject to catabolite repression by glucose^{4, 19, 110}. Conserved catabolite-responsive elements (*cre*), which are binding sequences for CcpA (catabolite control protein A) of Gram-positive bacteria, have been identified in the *arcA* promoter regions of *S. gordonii* and *Enterococcus faecalis*^{4, 19}.

Some halobacteria species of *Archaea*, such as *H. salinarium* (formerly *H. halobium*), have the ADI pathway for fermentative arginine degradation⁸¹. The *arcACB* genes of *H. salinarium* cluster, but they are separately transcribed from their own promoters⁸¹. The upstream *arcR* gene, located in the same orientation as *arcACB*, encodes a putative regulatory protein of the IclR family. The involvement and regulatory mechanism of the putative regulatory gene in anaerobic *arcACB* expression requires elucidation.

6.2. CbrA-CbrB Two-Component Regulatory System

Two-component regulatory systems, consisting of a sensor/histidine kinase (also called a transmitter) and a cognate response regulator, are the paradigm of bacterial adaptation to changes in environmental status such as nutrient availability, oxygen tension, osmolarity, and cell population (for reviews, see the articles in refs [20] and [37]). Environmental signals can trigger autophosphorylation of the transmitter in the histidine kinase domain through the sensor domain. Subsequent transfer of the phosphate to the receiver domain of the response regulator results in activation of the regulator protein³⁷. The active form of the response regulator then induces expression of the genes necessary for adaptation to environmental changes. The *cbrAB* genes were identified as being essential for the utilization of arginine or ornithine as carbon sources⁷⁰. In an independent study, the same genes were found as positive control elements required for σ^{54} -dependent expression of the lipase (*lipAH*) genes and designated *lipQR*²⁶. The putative sensor domain of CbrA having 12 possible transmembrane helices at the amino-terminal half (residues 1–490) has about 20% identity with sodium/solute symporters, and the histidine kinase domain at the carboxyl-terminal part (residues 730–983) shares 34% identity with *E. coli* NtrB (also called NRII or GlnG). The CbrB response regulator resembles (45% identity) NtrC (NRI or GlnL), which is the cognate response regulator of NtrB that modulates the nitrogen-responsive expression of *glnA* (encoding glutamine synthetase) and other nitrogen assimilation genes via the σ^{54} -RNA polymerase holoenzyme in enteric bacteria^{37, 55, 64, 87}. The CbrA-CbrB pair is structurally similar to, but functionally distinct from, the NtrB-NtrC pair; in addition, *P. aeruginosa* PAO1 has NtrB and NtrC that appear to be structurally and functionally equivalent to the *E. coli* counterparts (<http://www.pseudomonas.com>).

Mutations of *cbrA* or *cbrB* impair the utilization of a wide range of compounds including amino acids (proline, alanine, histidine, ornithine, and arginine), polyamines (putrescine, spermidine, and spermine), as well as agmatine, gluconate, and citrate^{52, 70} (Figure 6). Such growth phenotypes of the mutants coincide with that of an *rpoN* mutant lacking σ^{54} -factor, further supporting the notion that CbrB is a transcription activator of σ^{54} -promoters. In *cbrA* or *cbrB* mutants, the *aot-argR* operon is not expressed in the presence of arginine (thereby, the *aru* operon is not activated) and the *hut* (previously called *hiu*) histidine utilization operon is also not expressed⁷⁰. In contrast, the *aguBA* and *putAP* (= *pru*) proline utilization operons are normally expressed^{66, 70}. The inability to utilize agmatine and polyamines is due to a failure of *spu* operon expression, abolishing the formation of putrescine transaminase and the spermidine and spermine transport system (see above).

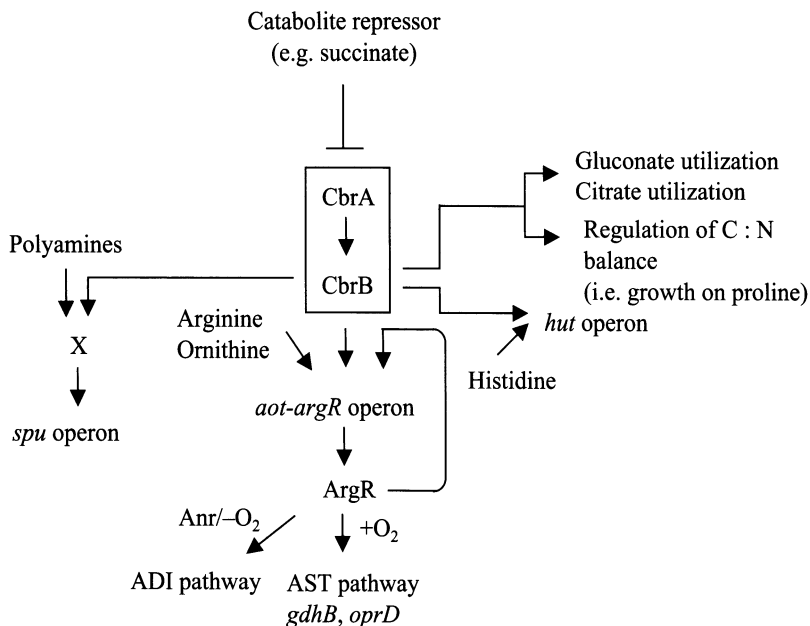


Figure 6. Regulation of arginine and polyamine metabolism and other metabolic functions by the CbrA-CbrB two-component regulatory system. The current model assumes that under carbon-poor conditions CbrB becomes active (CbrB-P) through phosphotransfer from CbrA. When arginine is present, the *aot-argR* operon is expressed to promote uptake of the amino acid and ArgR synthesis, leading to induction of the AST pathway and other arginine-regulated genes. Histidine relieves repression of the *hutU* operon mediated by the HutC repressor¹ and CbrB-P activates expression of the *hut* operon. Polyamines induce a transcription activator for the *spuABCDEFGH* operon with the help of CbrB-P⁵². Under carbon-poor conditions, CbrB-P maintains a healthy balance of C and N by activating an as yet unknown metabolic enzyme(s). Failure of the C:N control might cause the observed growth defects on proline⁷⁰. Roles of the two-component system in glucose and gluconate utilization are unknown.

A model describing how CbrB controls the expression of these catabolic operons has the following features (Figure 6). The arginine-inducible P2 promoter of the *aot-argR* operon and the *spuA* promoter of the *spuABCDEFGH* operon appear to be σ^{70} -dependent^{52, 71}. Such promoter features imply that CbrB indirectly modulates these promoters by means of transcription activators of σ^{70} -RNA polymerase, similarly, NtrC controls σ^{70} -dependent nitrogen-related genes such as *put*, *hut*, *gdh* (glutamate dehydrogenase), and *ure* (urease) via the NAC protein in enteric bacteria^{37, 55}. During growth on polyamines, PAO1 cells, but not *cbrB* mutant cells, express a protein, which can bind to the *spuA* promoter⁵² and which might be an intermediate

regulator. However, so far no indication has been obtained that suggests the presence of a protein required for activation of the P2 promoter other than CbrA-CbrB and ArgR. And no positive regulator appears to be required for *hut* expression, as a *hutC* mutant devoid of the repressor fully expresses the operon in the absence of the inducer^{1,70}. Clearly, a more detailed analysis is warranted for elucidation of the role of CbrB in the transcription mechanisms of these promoters.

The signal for CbrA might not be compounds metabolized by the relevant pathways. CbrA and CbrB functions are required for utilization of the amino acids and polyamines only as carbon sources. This implies that CbrB may become active when a preferred carbon source (i.e., a TCA cycle intermediate such as succinate) is absent (Figure 6). The membrane sensor of CbrA might sense concentrations of a TCA cycle intermediate(s) that reflects the availability of a carbon source to control autophosphorylation of the kinase domain. Alternatively, by analogy with the NtrB-NtrC system where PII (the *glnB* product) controls the amounts of the phosphorylated form of NtrC by modulating the phosphatase activity of NtrB, depending on the cellular concentrations of 2-ketoglutarate and glutamine³⁷, a PII-like protein might modulate phosphorylation of CbrB. These hypotheses require verification.

In addition to the regulation of specific pathways, CbrAB appears to play an important role in maintenance of a cellular C:N balance (Figure 6). Although CbrA or CbrB mutants can normally express the PutA (=PruAB) protein (bifunctional proline dehydrogenase/proline 5-carboxylate dehydrogenase⁶⁶ [also see Chapter 10, Volume 3], which converts proline into glutamate), the *cbrA* and *cbrB* mutants cannot proliferate on proline. Succinate fully restores impaired growth of the mutants on proline at =1 mM, a concentration that is not sufficient to serve as carbon source, whereas 20 mM ammonia antagonizes the growth promoted by succinate⁷⁰. *Escherichia coli* and some other enteric bacteria utilize amino acids as the sole nitrogen source and regulate amino acid metabolism by NtrB-NtrC in response to ammonia availability⁵⁵. *Pseudomonas aeruginosa* PAO1 utilizes arginine/ornithine and histidine as the sole source of carbon and nitrogen. During growth on amino acids as carbon and nitrogen sources, *P. aeruginosa* PAO1 generates and secretes large amounts of ammonia into the medium to maintain an optimal C:N balance. CbrA-CbrB might control a specific function that is involved in the control of C:N balance such as ammonia secretion. Whether the growth defect of *cbrA* or *cbrB* mutant on alanine as a carbon and nitrogen source and on gluconate and citrate as carbon sources with ammonia as a nitrogen source is due to the same problem or due to the absence of expression of the corresponding catabolic enzymes remains to be explored.

7. CONCLUDING REMARKS

Owing to the presence of four arginine catabolic pathways, *P. aeruginosa* PAO1 can utilize arginine as an energy source under anaerobic conditions, as carbon, energy, and nitrogen sources under aerobic conditions, and as a precursor of putrescine biosynthesis. The anaerobic ADI pathway and aerobic AST pathways are controlled by the anaerobic Anr regulator and by the CbrA-CbrB two-component regulatory system, respectively. These pathway genes are transcribed as single transcription units, allowing simultaneous regulation of the pathways. The ADH pathway functions as an arginine catabolic pathway for carbon and nitrogen sources in *P. putida* and as a D-arginine catabolic route in *P. aeruginosa*. This pathway consists of several distinct transcriptional units that are cumulatively induced by pathway intermediates, allowing the utilization of exogenous intermediates.

The metabolic pathway network (Figures 1 and 2) allows *P. aeruginosa* PAO1 to utilize over 20 compounds as carbon and/or nitrogen sources. This network consists of a minimal number of catabolic enzymes since the KauB aldehyde dehydrogenase catalyzes three reactions and the AguAB enzymes of the ADC pathway perform both agmatine catabolism and putrescine biosynthesis.

The σ^{54} RNA polymerase holoenzyme is necessary for the formation of pili and flagella and expresses various functions necessary for environmental adaptation including catabolism of phenolic compound, amino acids, and polyamines in *P. aeruginosa* PAO1^{33, 38, 92}. It is also involved in controlling nitrogen assimilation and alginate (extracellular polysaccharide) synthesis⁶². Thus, the σ^{54} -holoenzyme of *Pseudomonas* spp. appears to express a wider range of functions than that of enteric bacteria. Arginine/ornithine metabolism and polyamine metabolism are controlled by the CbrA-CbrB two-component system, in response to a signal of carbon availability. Presumably, this two-component system could activate the expression of metabolic pathways when preferred carbon sources are not available and, when a preferred carbon source is present, also act as a catabolite regulator (Figure 6). Understanding the signal for the transmitter and the regulatory mechanism of the response regulator will afford further insight into the catabolite control of amino acid and polyamine metabolism in pseudomonads³².

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REFERENCES

1. Allison, S.L. and Phillips, A.T., 1990, Nucleotide sequence of the gene encoding the repressor for the histidine utilization genes of *Pseudomonas putida*. *J. Bacteriol.*, 172:5470–5476.
2. Arai, H., Igarashi, Y., and Kodama, T., 1995, Expression of the *nir* and *nor* genes for denitrification of *Pseudomonas aeruginosa* requires a novel CRP/FNR-related transcriptional regulator, DNR, in addition to ANR. *FEBS Lett.*, 371:73–76.
3. Arai, H., Kodama, T., and Igarashi, Y., 1997, Cascade regulation of the two CRP/FNR-related transcriptional regulators (ANR and DNR) and the denitrification enzymes in *Pseudomonas aeruginosa*. *Mol. Microbiol.*, 25:1141–1148.
4. Barcelona-Andres, B., Marina, A., and Rubio, V., 2002, Gene structure, organization, expression, and potential regulatory mechanisms of arginine catabolism in *Enterococcus faecalis*. *J. Bacteriol.*, 184:6289–6300.
5. Bartsch, K., von Johnn-Marteville, A., and Schulz, A., 1990, Molecular analysis of two genes of the *Escherichia coli* *gab* cluster: Nucleotide sequence of the glutamate:succinic semialdehyde transaminase gene (*gabT*) and characterization of the succinic semialdehyde dehydrogenase gene (*gabD*). *J. Bacteriol.*, 172:7035–7042.
6. Baur, H., Tricot, C., Stalon, V., and Haas, D., 1990, Conversion of catabolic ornithine carbamoyltransferase to an anabolic enzyme. *J. Biol. Chem.*, 265:14728–14731.
7. Belitsky, B.R., 2002, Biosynthesis of amino acids of the glutamate and aspartate family, alanine, and polyamines. In A.L. Sonenshein, J.A. Hoch, and R. Losick (eds), *Bacillus subtilis and Its Closest Relatives: From Genes to Cells*, pp. 203–231. ASM Press, Washington DC.
8. Belitsky, B.R. and Sonenshein, A.L., 2002, GabR, a member of a novel protein family, regulates the utilization of γ -aminobutyrate in *Bacillus subtilis*. *Mol. Microbiol.*, 45:569–583.
9. Bourdineaud, J.-P., Heierli, D.H., Gamper, M., Verhoogt, H.J., Driessen, A.J., Konings, W.N., Lazdunski, C., and Haas, D., 1993, Characterization of the *arcD* arginine:ornithine exchanger of *Pseudomonas aeruginosa*. Localization in the cytoplasmic membrane and a topological model. *J. Biol. Chem.*, 268:5417–5424.
10. Boyle, S.M., Barroso, L., Moore, R.C., Wright, J.M., and Patel, T., 1994, Primary structure of the *speC* gene encoding biosynthetic ornithine decarboxylase in *Escherichia coli*. *Gene*, 151:157–160.
11. Cohen, S., 1998, *A Guide to the Polyamines*. Oxford University Press, Oxford.
12. Chou, C.S. and Rodwell, V.W., 1972, Metabolism of basic amino acids in *Pseudomonas putida*. γ -guanidinobutyrate amidohydrolase. *J. Biol. Chem.*, 247:4486–4490.
13. Cui, S., Meng, J., and Bhagwat, A.A., 2001, Availability of glutamate and arginine during acid challenge determines cell density-dependent survival phenotype of *Escherichia coli* strains. *Appl. Environ. Microbiol.*, 67:4914–4918.
14. Cunin, R., Glandsdorff, N., Piérard, A., and Stalon, V., 1986, Biosynthesis and metabolism of arginine in bacteria. *Microbiol. Rev.*, 50:314–352.
15. Davis, R.H., Morris, D.R., and Coffino, P., 1992, Sequestered end products and enzyme regulation: The case of ornithine decarboxylase. *Microbiol. Rev.*, 56:280–290.
16. D'Hooghe, I., Vander Wauven, C., Michielis, J., Tricot, C., De Wilde, P., Vanderleyden, J., and Stalon, V., 1997, The arginine pathway in *Rhizobium etli*: DNA sequence analysis and functional study of the *arcABC* genes. *J. Bacteriol.*, 179:7403–7409.

17. De Angelis, M., Mariotti, L., Rossi, J., Servili, M., Fox, P.F., Rollan, G., and Gobbetti, M., 2002, Arginine catabolism by sourdough lactic acid bacteria: Purification and characterization of the arginine deiminase pathway enzymes from *Lactobacillus sanfranciscensis* CB1. *Appl. Environ. Microbiol.*, 68:6193–6201.
18. Donald, L.J., Molgat, G.F., and Duckworth, H.W., 1989, Cloning, sequence, and expression of the gene for NADH-sensitive citrate synthase of *Pseudomonas aeruginosa*. *J. Bacteriol.*, 171:5542–5550.
19. Dong, Y., Chen, Y.Y., Snyder, J.A., and Burne, R.A., 2002, Isolation and molecular analysis of the gene cluster for the arginine deiminase system from *Streptococcus gordonii* DL1. *Appl. Environ. Microbiol.*, 68:5549–5553.
20. Dunny, G.M. and Winans, S.C. (eds), 1999, *Cell-Cell Signaling in Bacteria*. American Society for Microbiology Press, Washington, DC.
21. Egan, S.M., 2002, Growing repertoire of AraC/XylS activators. *J. Bacteriol.*, 184:5529–5532.
22. Fraley, C.D., Kim, J.H., McCann, M.P., and Matin, A., 1998, The *Escherichia coli* starvation gene *cstC* is involved in amino acid catabolism. *J. Bacteriol.*, 180:4287–4290.
23. Galimand, M., Gamper, M., Zimmermann, A., and Haas, D., 1991, Positive FNR-like control of anaerobic arginine degradation and nitrate respiration in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 173:1598–1606.
24. Gallegos, M.T., Schleif, R., Bairoch, A., Hofmann, K., and Ramos, J.L., 1997, AraC/XylS family of transcriptional regulators. *Microbiol. Mol. Biol. Rev.*, 61:393–410.
25. Gamper, M. and Haas, D., 1993, Processing of the *Pseudomonas aeruginosa* *arcDABC* mRNA requires functional RNase E in *Escherichia coli*. *Gene*, 129:119–122.
26. Gerritse, G. and Quax, W.J., 2001, Expression system for altered expression levels. US Patent No. 6,313,283 B1.
27. Glansdorff, N., 1996, Biosynthesis of arginine and polyamines. In F.C. Neidhardt, R. Curtiss III, J.L. Ingraham, M. Schaechter, and H.E. Umbarger (eds), *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, pp. 408–433. ASM Press, Washington, DC.
28. Haas, D., Galimand, M., Gamper, M., and Zimmermann, A., 1990, Arginine network of *Pseudomonas aeruginosa*: Specific and global controls. In S. Silver, A.M. Chakraborty, B. Iglewski, and S. Kaplan (eds), *Pseudomonas: Biotransformations, Pathogenesis, and Evolving Biotechnology*, pp. 303–316. ASM Press, Washington DC.
29. Haas, D., Holloway, B.W., Schamböck, A., and Leisinger, T., 1977, The genetic organization of arginine biosynthesis in *Pseudomonas aeruginosa*. *Mol. Gen. Genet.*, 154:7–22.
30. Haas, D., Matsumoto, H., Moretti, P., Stalon, V., and Mercenier, A., 1984, Arginine degradation in *Pseudomonas aeruginosa* mutant blocked in two arginine catabolic pathways. *Mol. Gen. Genet.*, 193:437–444.
31. Hasegawa, N., Arai, H., and Igarashi, Y., 1998, Activation of a consensus FNR-dependent promoter by DNR of *Pseudomonas aeruginosa* in response to nitrite. *FEMS Microbiol. Lett.*, 166:213–217.
32. Hester, K.L., Lehman, J., Najar, F., Song, L., Roe, B.A., MacGregor, Hager, P.W., Phibbs, P.V., Jr, and Sokatch, J.R., 2000, Crc is involved in catabolite repression control of the *bkd* operon of *Pseudomonas putida* and *Pseudomonas aeruginosa*. *J. Bacteriol.*, 182:1144–1149.
33. Heurlier, K., Dénervaud, V., Pessi, G., Reimann, C., and Haas, D., 2003, Negative control of quorum sensing by RpoN (σ^{54}) in *Pseudomonas aeruginosa* PAO1. *J. Bacteriol.*, 185:2227–2235.
34. Hisano, T., Abe, S., Wakashiro, M., Kimura, A., and Murata, K., 1990, Microbial spermidine dehydrogenase: Purification and properties of the enzyme in *Pseudomonas aeruginosa* and *Citrobacter freundii*. *J. Ferment. Bioeng.*, 69:335–340.

35. Hisano, T., Murata, K., Kimura, A., Matsushita, K., and Adachi, O., 1992, Further properties of spermidine dehydrogenase from *Citrobacter freundii* IFO 12681. *Biosci. Biotech. Biochem.*, 56:311–314.
36. Hisano, T., Murata, K., Kimura, A., Matsushita, K., Toyama, H., and Adachi, O., 1992, Characterization of membrane-bound spermidine dehydrogenase of *Citrobacter freundii*. *Biosci. Biotech. Biochem.*, 56:1916–1920.
37. Hoch, J.A. and Silhavy, T.J. (eds), 1995, *Two-Component Signal Transduction*. American Society for Microbiology Press, Washington, DC.
38. Ishimoto, K.S. and Lory, S., 1989, Formation of pilin in *Pseudomonas aeruginosa* requires the alternative σ factor (RpoN) of RNA polymerase. *Proc. Natl. Acad. Sci. USA*, 86:1954–1957.
39. Itoh, Y., 1997, Cloning and characterization of the *aru* genes encoding enzymes of the catabolic arginine succinyltransferase pathway in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 179:7280–7290.
40. Itoh, Y., Soldati, L., Stalon, V., Falmagne, P., Terawaki, Y., Leisinger, T., and Haas, D., 1988, Anabolic ornithine carbamoyltransferase of *Pseudomonas aeruginosa*: Nucleotide sequence and transcriptional control of the *argF* structural gene. *J. Bacteriol.*, 170:2725–2734.
41. Jann, A., Matsumoto, H., and Haas, D., 1988, The fourth arginine pathway of *Pseudomonas aeruginosa*. *J. Gen. Microbiol.*, 134:1043–1053.
42. Jann, A., Stalon, V., Vander Wauven, C., Leisinger, T., and Haas, D., 1986, N^2 -Succinylated intermediates in an arginine catabolic pathway of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA*, 83:4937–4941.
43. Ka, J.O., Urbance, J., Ye, R.W., Ahn, T.Y., and Tiedje, J.M., 1997, Diversity of oxygen and N-oxide regulation of nitrite reductase in denitrifying bacteria. *FEMS Microbiol. Lett.*, 156:55–60.
44. Kiupakis, A.K. and Reitzer, L., 2002, ArgR-independent induction and ArgR-dependent superinduction of the *astCADBE* operon in *Escherichia coli*. *J. Bacteriol.*, 184:2940–2950.
45. Klingel, U., Miller, C.M. North, A.K., Stockley, P.G., and Baumberg, S., 1995, A binding site for activation by the *Bacillus subtilis* AhrC protein, a repressor/activator of arginine metabolism. *Mol. Gen. Genet.*, 248:329–340.
46. Krieger, R., Rompf, A., Schobert, M., and Jahn, D., 2002, The *Pseudomonas aeruginosa* *hemA* promoter is regulated by Anr, Dnr, NarL and integration host factor. *Mol. Genet. Genomics*, 267:409–417.
47. Kwon, D.H., Lu, C.D., Walthall, D.A., Brown, T.M., Houghton, J.E., and Abdelal, A.T., 1994, Structure and regulation of the *carAB* operon of *Pseudomonas aeruginosa* and *Pseudomonas stutzeri*: No untranslated region exists. *J. Bacteriol.*, 176:2532–2542.
48. Laville, J., Blumer, C., Von Schroetter, C., Gaia, V., Defago, G., Keel, C., and Haas, D., 1998, Characterization of the *hcnABC* gene cluster encoding hydrogen cyanide synthase and anaerobic regulation by ANR in the strictly aerobic biocontrol agent *Pseudomonas fluorescens* CHA0. *J. Bacteriol.*, 180:3187–3196.
49. Lu, C.D. and Abdelal, A.T., 1999, Role of ArgR in activation of the *ast* operon, encoding enzymes of the arginine succinyltransferase pathway in *Salmonella typhimurium*. *J. Bacteriol.*, 181:1934–1938.
50. Lu, C.D., Winteler, H., Abdelal, A., and Haas, D., 1999, The ArgR regulatory protein, a helper to the anaerobic regulator ANR during transcriptional activation of the *arcD* promoter in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 181:2459–2464.
51. Lu, C.D. and Abdelal, A.T., 2001, The *gdhB* gene of *Pseudomonas aeruginosa* encodes an arginine-inducible NAD^+ -dependent glutamate dehydrogenase which is subject to allosteric regulation. *J. Bacteriol.*, 183:490–499.

52. Lu, C.D., Itoh, Y., Nakada, Y., and Jiang, Y., 2002, Functional analysis and regulation of the divergent *spuABCDEFGH-spuI* operons for polyamine uptake and utilization in *Pseudomonas aeruginosa* PAO1. *J. Bacteriol.*, 184:3765–3773.
53. Lüthi, E., Baur, H., Gamper, M., Brunner, F., Villeval, D., Mercenier, A., and Haas, D., 1990, The *arc* operon for anaerobic arginine catabolism in *Pseudomonas aeruginosa* contains an additional gene, *arcD*, encoding a membrane protein. *Gene*, 87:37–43.
54. Lynch, A.S. and Lin, E.C.C., 1996, Responses to molecular oxygen. In F.C. Neidhardt, R. Curtiss III, J.L. Ingraham, M. Schaechter, and H.E. Umbarger (eds), *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, pp. 1526–1538. American Society for Microbiology Press, Washington, DC.
55. Magasanik, B., 1996, Regulation of nitrogen utilization, pp. 1344–1356. In F.C. Neidhardt, R. Curtiss III, J.L. Ingraham, M. Schaechter, and H.E. Umbarger (eds), *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn. American Society for Microbiology Press, Washington, DC.
56. Maghnouj, A., Abu-Bakr, A.A., Baumberg, S., Stalon, V., and Vander Wauven, C., 2000, Regulation of anaerobic arginine catabolism in *Bacillus licheniformis* by a protein of the Crp/Fnr family. *FEMS Microbiol. Lett.*, 191:227–234.
57. Maghnouj, A., de Sousa Cabral, T.F., Stalon, V., and Vander Wauven, C., 1998, The *arcABDC* gene cluster, encoding the arginine deiminase pathway of *Bacillus licheniformis*, and its activation by the arginine repressor *argR*. *J. Bacteriol.*, 180:6468–6475.
58. Marcq, S., Diaz-Ruano, A., Charlier, P., Dideberg, O., Tricot, C., Piérard, A., and Stalon, V., 1991, Molecular size and symmetry of *Pseudomonas aeruginosa* catabolic ornithine carbamoyltransferase: An X-ray crystallography analysis. *J. Mol. Biol.*, 220:9–12.
59. Marquis, R., Bender, G.R., Murray, D.R., and Wong, A., 1987, Arginine deiminase system and bacterial adaptation to acid environments. *Appl. Environ. Microbiol.*, 53:198–200.
60. Mercenier, A., Simon, J.-P., Haas, D., and Stalon, V., 1980, Catabolism of L-arginine by *Pseudomonas aeruginosa*. *J. Gen. Microbiol.*, 116:381–389.
61. Miller, D.L. and Rodwell, V.W., 1971, Metabolism of basic amino acids in *Pseudomonas putida*. Intermediates in L-arginine metabolism. *J. Biol. Chem.*, 246:5053–5058.
62. Mohr, C.D., Martin, D.W., Konyecsni, W.M., Govan, J.R.W., Lory, S., and Deretic, V., 1990, Role of the far-upstream sites of the *algD* promoter and the *algR* and *rpoN* genes in environmental modulation of mucoidy in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 172:6576–6580.
63. Mouz, N., Tricot, C., Ebel, C., Petillot, Y., Stalon, V., and Dideberg, O., 1996, Use of a designed fusion protein dissociates allosteric properties from the dodecameric state of *Pseudomonas aeruginosa* catabolic ornithine carbamoyltransferase. *Proc. Natl. Acad. Sci. USA*, 93:9414–9419.
64. Nixon, B.T., Ronson, C.W., and Ausubel, F.M., 1986, Two-component regulatory systems responsive to environmental stimuli share strong conserved domains with the nitrogen assimilation regulatory genes *ntrB* and *ntrC*. *Proc. Natl. Acad. Sci. USA*, 83:7850–7854.
65. Nakada, Y. and Itoh, Y., 2002, Characterization and regulation of the *gbuA* gene, encoding guanidinobutyrase in the arginine dehydrogenase pathway of *Pseudomonas aeruginosa* PAO1. *J. Bacteriol.*, 184:3377–3384.
66. Nakada, Y. and Itoh, Y., 2002, Divergent structure and regulation mechanism of proline catabolic systems: Characterization of the *putAP* proline catabolic operon of *Pseudomonas aeruginosa* PAO1 and its regulation by PutR, an AraC/XylS family protein. *J. Bacteriol.*, 185:5633–5640.
67. Nakada, Y. and Itoh, Y., 2003, Identification of the putrescine biosynthetic genes in *Pseudomonas aeruginosa* and characterization of agmatine deiminase and N-carbamolputrescine amidohydrolase of the arginine decarboxylase pathway. *Microbiology*, 149:707–714.

68. Nakada, Y., Jiang, Y., Nishijyo, T., Itoh, Y., and Lu, C.D., 2001, Molecular characterization and regulation of the *aguBA* operon, responsible for agmatine utilization in *Pseudomonas aeruginosa* PAO1. *J. Bacteriol.*, 183:6517–6524.
69. Niegemann, E., Schulz, A., and Bartsch, K., 1993, Molecular organization of the *Escherichia coli* *gab* cluster: Nucleotide sequence of the structural genes *gabD* and *gabP* and expression of the GABA permease gene. *Arch. Microbiol.*, 160:454–460.
70. Nishijyo, T., Haas, D., and Itoh, Y., 2001, The CbrA-CbrB two-component regulatory system controls the utilization of multiple carbon and nitrogen sources in *Pseudomonas aeruginosa*. *Mol. Microbiol.*, 40:917–931.
71. Nishijyo, T., Park, S.M., Lu, C.D., Itoh, Y., and Abdelal, A.T., 1998, Molecular characterization and regulation of an operon encoding a system for transport of arginine and ornithine and the ArgR regulatory protein in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 180:5559–5566.
72. Nguyen, V.T., Baker, D.P., Tricot, C., Baur, H., Villeret, V., Dideberg, O., Gigot, D., Stalon, V., and Haas, D., 1996, Catabolic ornithine carbamoyltransferase of *Pseudomonas aeruginosa*: Importance of the N-terminal region for dodecameric structure and homotropic carbamoylphosphate cooperativity. *Eur. J. Biochem.*, 236:283–293.
73. Nguyen, V.T., Tricot, C., Stalon, V., Dideberg, O., Villeret, V., and Haas, D., 1994, Methionine-321 in the C-terminal α -helix of catabolic ornithine carbamoyltransferase from *Pseudomonas aeruginosa* is important for positive homotropic cooperativity. *FEMS Microbiol. Lett.*, 124:411–418.
74. Ochs, M.M., Lu, C.-D., Hancock, R.W., and Abdelal, A.T., 1999, Amino acid-mediated induction of the basic amino acid-specific outer membrane protein OprD from *Pseudomonas aeruginosa*. *J. Bacteriol.*, 181:5426–5432.
75. Ohtani, K., Bando, M., Swe, T., Banu, S., Oe, M., Hayashi, H., and Shimizu, T., 1997, Collagenase gene *colA* is located in the 3'-flanking regions of the perfringolysin O *pfoA* locus in *Clostridium perfringens*. *FEMS Microbiol. Lett.*, 146:155–159.
76. Park, S.M., Lu, C.D., and Abdelal, A.T., 1997, Cloning and characterization of *argR*, a gene that participates in regulation of arginine biosynthesis and catabolism in *Pseudomonas aeruginosa* PAO1. *J. Bacteriol.*, 179:5300–5308.
77. Park, S.M., Lu, C.D., and Abdelal, A.T., 1997, Purification and characterization of an arginine regulatory protein, ArgR, from *Pseudomonas aeruginosa* and its interactions with the control regions for the *car*, *argF*, and *aru* operon. *J. Bacteriol.*, 179:5309–5317.
78. Pessi, G. and Haas, D., 2000, Transcriptional control of the hydrogen cyanide biosynthetic genes *hcnABC* by the anaerobic regulator ANR and the quorum-sensing regulators LasR and RhIR in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 182:6940–6949.
79. Piotrowski, M., Janowitz, T., and Kneifel, H., 2003, Plant C-N hydrolases and the identification of a plant N-carbamoylputrescine amidohydrolase involved in polyamine biosynthesis. *J. Biol. Chem.*, 278:1708–1712.
80. Price, T., French, G.L., Talsamia, H., and Phillips, L., 1986, Differentiation of *Streptococcus sanguis* and *S. mitior* by whole-cell rhamnose content and possession of arginine dihydrolase. *J. Med. Microbiol.*, 21:189–197.
81. Ruepp, A. and Soppa, J., 1996, Fermentative arginine degradation in *Halobacterium salinarum* (formerly *Halobacterium halobium*): Genes, gene products, and transcripts of the *arcRACB* gene cluster. *J. Bacteriol.*, 178:4942–4947.
82. Ray, A. and Williams, H.D., 1997, The effects of mutation of the *anr* gene on the aerobic respiratory chain of *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.*, 156:227–232.
83. Rompf, A., Hungerer, C., Hoffmann, T., Lindenmeyer, M., Romling, U., Gross, U., Doss, M.O., Arai, H., Igarashi, Y., and Jahn, D., 1998, Regulation of *Pseudomonas aeruginosa* *hemF* and *hemN* by the dual action of the redox response regulators Anr and Dnr. *Mol. Microbiol.*, 29:985–997.

84. Samsonova, N., Smirnov, S.V., Altman, I.B., and Ptitsyn, L.R., 2003, Molecular cloning and characterization of *Escherichia coli* K12 *ygiG* gene. *BMC Microbiol.*, 3:2.
85. Schneider, B.L., Kiupakis, A.K., and Reitzer, L.J., 1998, Arginine catabolism and the arginine succinyltransferase pathway in *Escherichia coli*. *J. Bacteriol.*, 180:4278–4286.
86. Schneider, B.L., Rugack, S., Kiupakis A.K., Kasbarian, H., Pybus C., and Reitzer, L., 2002, The *Escherichia coli* *gabDTPC* operon: Specific γ -aminobutyrate catabolism and non-specific induction. *J. Bacteriol.*, 184:6967–6986.
87. Shingler, V., 1996, Signal sensing σ^{54} -dependent regulators: Derepression as a control mechanism. *Mol. Microbiol.*, 19:409–416.
88. Stover, C.V. *et al.*, 2000, Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature*, 406:959–964.
89. Tabor, C.W. and Kellogg, P.D., 1970, Identification of flavin adenine dinucleotide and heme in a homogeneous spermidine dehydrogenase from *Serratia marcescens*. *J. Biol. Chem.*, 245:5424–5433.
90. Tobes, R. and Ramos, J.L., 2002, AraC-XylS database: A family of positive transcriptional regulators in bacteria. *Nucleic Acids Res.*, 30:318–321.
91. Tonon, T., Bourdineaud, P., and Louvaud-Funel, A., 2001, The *arcABC* gene cluster encoding the arginine deiminase pathway of *Oenococcus oeni*, and arginine induction of a CRP-like gene. *Res. Microbiol.*, 152:653–661.
92. Totten, P.A., Lara, J.C., and Lory, S., 1990, The *rpoN* gene product of *Pseudomonas aeruginosa* is required for expression of diverse genes, including the flagellin gene. *J. Bacteriol.*, 172:289–296.
93. Tricot, C., Nguyen, V.T., and Stalon, V., 1993, Steady-state kinetics and analysis of pH dependence on wild-type and a modified allosteric *Pseudomonas aeruginosa* ornithine carbamoyltransferase containing the replacement of glutamine 105 by alanine. *Eur. J. Biochem.*, 215:833–839.
94. Tricot, C., Schmid, S., Baur, H., Villeret, V., Dideberg, O., Haas, D., and Stalon, V., 1994, Catabolic ornithine carbamoyltransferase of *Pseudomonas aeruginosa*: Changes of allosteric properties resulting from modifications at the C-terminus. *Eur. J. Biochem.*, 221:555–561.
95. Tricot, C., Stalon, V., and Legrain, C., 1991, Isolation and characterization of *Pseudomonas putida* mutants affected in arginine, ornithine and citrulline catabolism: Function of the arginine oxidase and arginine succinyltransferase pathways. *J. Gen. Microbiol.*, 137:2911–2918.
96. Tricot, C., Vander Wauven, C., Wattiez, R., Falmagne, P., and Stalon, V., 1994, Purification and properties of a succinyltransferase from *Pseudomonas aeruginosa* specific for both arginine and ornithine. *Eur. J. Biochem.*, 224:853–861.
97. Tricot, C., Villeret, V., Sainz, G., Dideberg, O., and Stalon, V., 1998, Allosteric regulation in *Pseudomonas aeruginosa* catabolic ornithine carbamoyltransferase revisited: Association of concerted homotropic cooperative interactions and local heterotropic effects. *J. Mol. Biol.*, 283:695–704.
98. Vander Wauven, C., Jann, A., Haas, D., Leisinger, T., and Stalon, V., 1988, N^2 -Succinylornithine in ornithine catabolism of *Pseudomonas aeruginosa*. *Arch. Microbiol.*, 150:400–404.
99. Vander Wauven, C. and Stalon, V., 1985, Occurrence of succinyl derivatives in the catabolism of arginine in *Pseudomonas cepacia*. *J. Bacteriol.*, 164:882–886.
100. Vanderbilt, A.S., Gaby, N.S., and Rodwell, V.W., 1975, Intermediates and enzymes between α -ketoarginine and γ -guanidinobutyrate in the L-arginine catabolic pathway of *Pseudomonas putida*. *J. Biol. Chem.*, 250:5322–5329.
101. Vergès, M.C., Zuñiga, M., Morel-Deville, F., Pérez-Martínez, G., Zagorec, M., and Ehrlich, S.D., 1999, Relationships between arginine degradation, pH and survival in *Lactobacillus sakei*. *FEMS Microbiol. Lett.*, 180:297–304.

102. Verhoogt, H.J., Smit, H., Abee, T., Gamper, M., Driessen, A.J., Haas, D., and Konings, W.N., 1992, *arcD*, the first gene of the *arc* operon for anaerobic arginine catabolism in *Pseudomonas aeruginosa*, encodes an arginine-ornithine exchanger. *J. Bacteriol.* 174:1568–1573.
103. Vijgenboom, E., Busch, J.E., and Canters, G.W., 1997, *In vivo* studies disprove an obligatory role of azurin in denitrification in *Pseudomonas aeruginosa* and show that *azu* expression is under control of *rpoS* and ANR. *Microbiology*, 143:2853–2863.
104. Villeret, V., Tricot, C., Stalon, V., and Dideberg, O., 1995, Crystal structure of *Pseudomonas aeruginosa* catabolic ornithine transcarbamoylase at 3.0-Å resolution: A different oligomeric organization in the transcarbamoylase family. *Proc. Natl. Acad. Sci. USA*, 92:10762–10766.
105. Voellmy, R. and Leisinger, T., 1975, Dual role for *N*²-acetylornithine 5-aminotransferase from *Pseudomonas aeruginosa* in arginine biosynthesis and arginine catabolism. *J. Bacteriol.*, 122:799–809.
106. Voellmy, R. and Leisinger, T., 1976, Role of 4-aminobutyrate aminotransferase in the arginine metabolism of *Pseudomonas aeruginosa*. *J. Bacteriol.*, 128:722–729.
107. Vollack, K.U., Hartig, E., Korner, H., and Zumft, W.G., 1999, Multiple transcription factors of the FNR family in denitrifying *Pseudomonas stutzeri*: Characterization of four *fnr*-like genes, regulatory responses and cognate metabolic processes. *Mol. Microbiol.*, 31:1681–1694.
108. Yorifuji, T. and Sugai, I., 1978, 3-Guanidinopropionate amidinohydrolase and 4-guanidinobutyrate amidinohydrolase of *Pseudomonas aeruginosa* PAO1. *Agric. Biol. Chem.*, 42:1789–1790.
109. Ye, R.W., Haas, D., Ka, J.O., Krishnapillai, V., Zimmermann, A., Baird, C., and Tiedje, J.M., 1995, Anaerobic activation of the entire denitrification pathway in *Pseudomonas aeruginosa* requires Anr, an analog of Fnr. *J. Bacteriol.*, 177:3606–3609.
110. Zúñiga, M., Champomier-Verges, M., Zagorec, M., and Pérez-Martínez, G., 1998, Structural and functional analysis of the gene cluster encoding the enzymes of the arginine deiminase pathway of *Lactobacillus sake*. *J. Bacteriol.*, 180:4154–4159.

PROLINE AND LYSINE METABOLISM

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1. INTRODUCTION

Besides their function as building blocks for proteins, certain amino acids have other important physiological roles. Such is the case of the non-polar imino acid proline and of lysine, a basic amino acid. Proline is accumulated by bacterial cells during osmotic stress as one of the prevalent compatible solutes that act as osmoprotectants^{21, 39}. It is also a precursor in the biosynthesis of pyoluteorin, an antifungal compound produced by several *Pseudomonas fluorescens* strains^{35, 47}. Lysine is involved in pathogenicity mechanisms in *Pseudomonas syringae*, by conjugation of the amino acid with indole acetic acid (IAA). These IAA-lysine conjugates interfere with auxin metabolism in plants infected by *P. syringae*, leading to alterations in plant development¹⁰. Lysine also participates indirectly in survival of bacteria to low pH, a stress situation that triggers lysine decarboxylation to produce cadaverine, which reduces outer membrane permeability^{42, 43}.

Despite their obvious differences in terms of chemical structure, proline and lysine biosynthesis are both somewhat related to the arginine biosynthetic pathway, described in the previous chapter. As we will see, this connection involves certain common intermediaries (resulting in cross-feeding between pathways), enzymes with dual functionality, and enzymes that show a close evolutionary origin.

Both proline and lysine can be used by fluorescent *Pseudomonas* as nitrogen and carbon sources. They are among the most abundant amino acids

in seed and root exudates^{6, 53, 55}, and therefore they constitute one of the main sources of nutrients in the rhizosphere (the soil region surrounding and under the influence of plant roots), an environment where fluorescent *Pseudomonas* can preferentially be found (see the corresponding chapter in Volume 1 for details).

The metabolism of these two amino acids is relatively well characterized in different organisms. In *Pseudomonas*, the enzymology of the two catabolic pathways and the specific biochemistry of some of the enzymes involved have been established. The genetics of proline catabolism have also been studied to a certain extent. However, lysine catabolism is mostly unexplored at the genetic level and in terms of the regulatory mechanisms involved. With respect to the biosynthetic pathways, some of the reactions have been experimentally established, whereas others have to be deduced by comparison with what is known in other organisms.

2. PROLINE BIOSYNTHESIS

Synthesis of proline follows a similar pathway in all prokaryotes studied so far. Three enzymes, the products of the *proB*, *proA* and *proC* genes, are responsible for the conversion of L-glutamate into L-proline^{4, 11, 21, 36, 44}. Although proline biosynthesis has not been studied in depth in *Pseudomonas*, the existence of homologues of those three genes in these organisms supports the proposed route for proline biosynthesis shown in Figure 1. The first step, catalyzed by glutamate kinase (ProB), converts glutamate in γ -glutamyl phosphate, which is then transformed in glutamyl- γ -semialdehyde by action of a dehydrogenase (ProA). Glutamyl- γ -semialdehyde is in equilibrium with a cyclic compound, Δ^1 -pyrroline-5-carboxylic acid, which is converted into L-proline by action of the Δ^1 -pyrroline-5-carboxylate reductase encoded by *proC*.

Two other alternative pathways have been described, both resulting in the biosynthesis of proline from L-ornithine, an intermediate in the metabolism of arginine. Ornithine can be directly converted into proline by action of L-ornithine cyclodeaminase, an enzyme having cyclase and deaminase activities⁵⁰. The second alternative is the deamination of ornithine resulting in glutamyl- γ -semialdehyde/ Δ^1 -pyrroline-5-carboxylate, a reaction described in *Clostridium* and *Staphylococcus*^{16, 48}. Ornithine aminotransferase activity seems also to be present in *Pseudomonas*^{12, 45}. However, it is unlikely that these routes are the primary source for proline biosynthesis. This is supported by the fact that a *proA* mutant of *Pseudomonas aeruginosa* shows proline auxotrophy, a defect that can be complemented by growth on ornithine and citrate only when a secondary mutation leading to increased ornithine aminotransferase

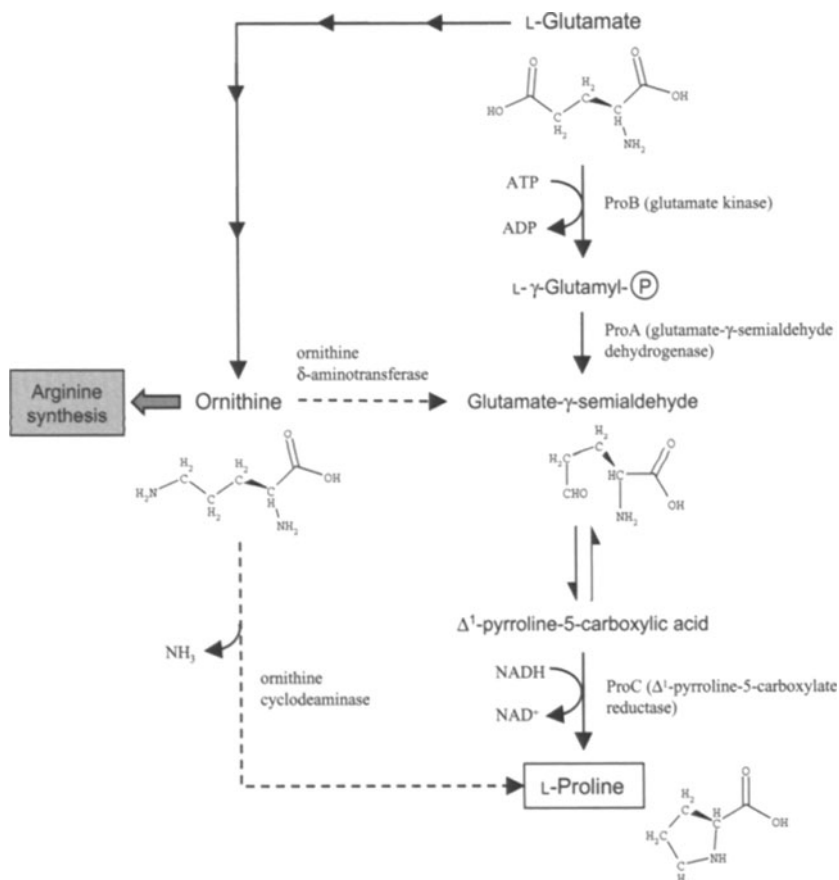


Figure 1. Proposed proline biosynthetic pathway(s) in *Pseudomonas*. Broken lines indicate activities that are secondary or not fully characterized in these organisms. For the sake of clarity, in this and all other figures, only some relevant chemical structures are shown. Chemical structures were drawn using the free ISIS Draw 2.4 software (MDL Information Systems, Inc.).

activity is introduced¹². Thus, this ornithine to proline pathway, via glutamyl-γ-semialdehyde seems to be accidental, and not a true source for proline biosynthesis. Ornithine cyclodeaminase activity has been reported in *P. putida*, where it has been proposed that proline is an intermediate in ornithine catabolism^{45, 50}. In the genome of *P. putida* KT2440, three genes coding for putative cyclodeaminases can be found, one of them (PP3533) very similar to known ornithine cyclodeaminases. It is also worth noting that in this strain there are two genes showing similarity to *proC*, although one of them is more closely related to *proC* homologues of different organisms. The existence of different possible enzymatic activities involved in the last step of proline biosynthesis,

either by the *pro* route or the ornithine cyclodeaminase pathway could account for the failure in different attempts at obtaining proline auxotrophs of *P. putida* KT2440 by random transposon mutagenesis (M.Espinosa-Urgel, unpublished). Such redundancy in metabolic pathways seems to be a hallmark of this bacterium, and may be a way to ensure key biosynthetic functions under any of the diverse environmental situations in which KT2440 thrives.

The regulation of proline biosynthesis has not been studied in detail and only a few data are available. The activity of the first enzyme, glutamate kinase, shows negative feedback by proline, but there is no transcriptional repression of the *proBA* operon by proline³⁶ in the case studied. In *P. aeruginosa*, there is an indirect effect of OruR, a regulator controlling ornithine utilization, which affects proline synthesis via the ornithine pathway¹². Also, given its role as an osmoprotectant, it could be postulated that changes in the medium osmolarity influence proline synthesis, as is the case in other organisms²¹. However, it is important to note that, contrary to many microorganisms, *proB* and *proA* are not organized as an operon in the *Pseudomonas* species sequenced to date, being located in separate regions of the chromosome. Thus, it is possible that the regulation of these two genes is also different in these organisms.

3. PROLINE TRANSPORT AND CATABOLISM

Proline transport is still poorly characterized at the molecular level in *Pseudomonas*. In other microorganisms, the way by which proline is incorporated into the cell varies depending on whether proline transport is the result of an osmotic stress situation or of proline being available as a primary nutrient. The differences begin at the outer membrane level. In *Escherichia coli* grown in low osmolarity media, the OmpF porin facilitates proline passage across the outer membrane and into the periplasm. An increase in the medium osmolarity causes the activation of the osmotic stress response, which leads to the overexpression of OmpC¹⁵. This porin also facilitates proline transport to the periplasm. From there, two osmolarity-dependent proline transporters, ProP and ProU, incorporate the amino acid into the cell. The structure of these permeases is different. ProP is a single integral membrane protein of the major facilitator superfamily (MFS), whereas ProU is an ABC transporter. Its components (an ATPase, an inner membrane protein and a periplasmic fusion protein) are encoded by the *proVWX* operon. In enteric bacteria, expression of *proP* and *proVWX* is induced under hyperosmotic conditions as well as by the stationary phase sigma factor σ^S (see refs [22], [28]). The osmotic stress response also leads to a blockage of proline catabolism, so that the amino acid does not enter a futile cycle of accumulation/degradation/biosynthesis and is accumulated as an osmoprotectant⁵.

The role of proline as an osmoprotectant in *Pseudomonas* awaits detailed investigation. At least two operons coding for proteins similar to the ProU transporter components can be found in all the sequenced *Pseudomonas*, and therefore it could be expected that osmolarity-dependent transport of proline across the cytoplasmic membrane proceeds in a way similar to that of enteric bacteria. However, significant differences may exist; proteins with some similarity to the MFS transporter ProP can be found in *Pseudomonas*, but only in *P. putida* a homologue very closely related to ProP proteins of enteric bacteria (over 80% identical residues) is present.

The main proline permease under non-stress conditions is the product of the *putP* gene. PutP is an integral membrane protein with 12 transmembrane domains acting as a high affinity proline/ Na^+ symporter. Other transport systems are likely to be active, at least in *P. putida*, since mutants deficient in expression of *putP* are still able to transport the imino acid, even though with lower efficiency than the wild type⁵³. It is worth noting the existence in *Pseudomonas* of a homologue of ProY, a cryptic proline transporter that has been described in *Salmonella typhimurium*¹⁹. Expression of the *proY* gene has not been studied in *Pseudomonas*. In *Salmonella*, it has only been observed when cloned in multicopy plasmids, and this overexpression can compensate *putP* mutations.

In *Pseudomonas*, as in many other microorganisms, proline is converted in two steps into glutamate by a bi-functional enzyme encoded by the *putA* gene (Figure 2). The PutA protein has the two activities required for this conversion, proline dehydrogenase and Δ^1 -pyrroline-5-carboxylate dehydrogenase^{26, 27, 53}. Two cofactors, FAD^{+2} and NAD^+ (or NADP^+) are required by the enzyme to catalyze each step, respectively.

Compared to the other metabolic pathways described in this chapter, the proline catabolic pathway is relatively well characterized in *Pseudomonas* at the genetic level. In *P. putida*, the *putA* gene is located in a cluster with *putP*. Both are divergently transcribed, as is the case in enteric bacteria, from a common intergenic regulatory region. Expression of the two genes is induced in the presence of proline and by root exudates^{53, 55}. The regulatory mechanism involves PutA, which in *P. putida* has a third, non-enzymatic activity, acting as a transcriptional repressor⁵⁴. In the absence of proline, PutA binds to the intergenic region between the *putA* and *putP* genes, blocking transcription from both promoters. The presence of proline prevents this interaction, resulting in the activation of the system⁵⁴. The enzymatic and regulatory functions of PutA are independent, at least in *P. putida*, since *putA* mutants unable to grow on proline but maintaining normal levels of expression of the *put* genes have been isolated⁵³. Expression of the two genes is also dependent on the sigma factor σ^{54} , involved in nitrogen regulation. However, since no σ^{54} -dependent promoter sequence can be found in the *put* regulatory region, it has been postulated

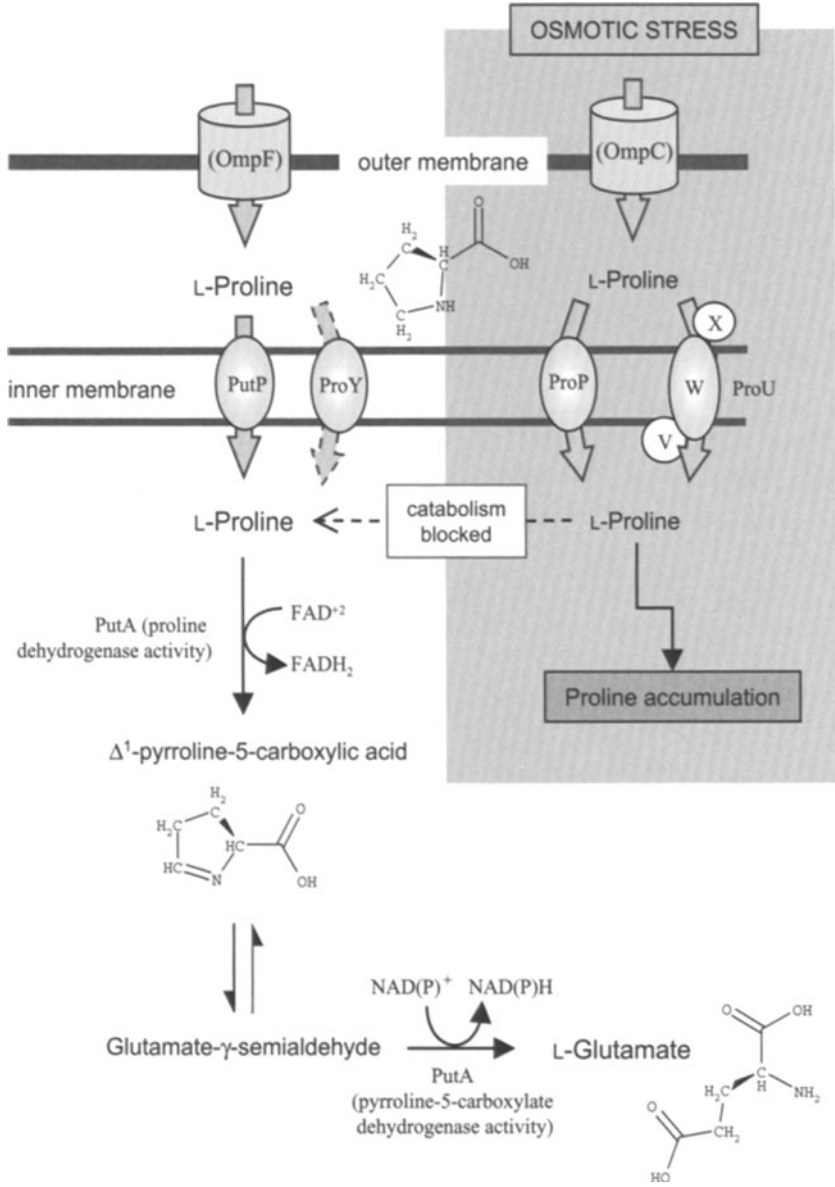


Figure 2. Proline transport and catabolic pathway. Note that the outer membrane proteins are shown in brackets, since they correspond to those that participate in proline acquisition by enteric bacteria. It remains to be determined what proteins play an equivalent role in *Pseudomonas*.

that σ^{54} would act indirectly via a transcriptional activator⁵⁴. A recent report indicates that a two-component regulatory system, CbrA-CbrB, which controls several carbon and nitrogen utilization pathways in *P. aeruginosa*, also affects proline utilization³⁴. The effect of *cbrA* or *cbrB* mutations on proline utilization is not direct and appears to be due to a metabolic block elsewhere that remains to be fully characterized³⁴. In *P. putida*, mutants affected in either element of a putative two-component regulatory system show reduced growth when proline is the only carbon and nitrogen source⁵³, but this potential regulator remains to be characterized in detail.

Interestingly, the genetic organization and regulation of the *put* genes in *P. putida* and *P. aeruginosa* PAO1 is very different³¹. In this bacterium, both genes form an operon controlled by PruR, a protein belonging to the AraC/XylS family of transcriptional regulators³¹, encoded by a gene located upstream of *putA*. PutA of *P. aeruginosa* does not have a regulatory activity and its sequence differs considerably from its *P. putida* counterpart. In fact, the protein lacks the carboxy-terminal region, which is responsible for the transcriptional regulatory activity of PutA in *P. putida*. Genes with some similarity to PruR can be found in the genomes of *P. putida*, *P. syringae* and *P. fluorescens*, but this similarity is relatively low (less than 35% identical residues).

Analysis of the genomes of the latter two organisms also reveals that the genetic organization of the *putA* and *putP* genes is identical to that in *P. putida*, with both genes adjacent and divergently oriented. This, and the fact that the PutA protein is very similar in sequence and identical in size in the three cases, suggests a common regulatory mechanism for proline catabolism, mediated by PutA in these bacteria, while *P. aeruginosa* constitutes the exception among *Pseudomonas*. It seems tempting to speculate that these differences are somehow a reflection of the different lifestyles between *P. aeruginosa* and the other three organisms. However, such correlation is not clear. Although in the plant symbiont *Sinorhizobium meliloti* PutA also controls its synthesis, in other soil and plant-associated bacteria such as *Agrobacterium tumefaciens* or *Bradyrhizobium japonicum* PutA is not self-regulated. In contrast, in enteric bacteria like *Salmonella* or *E. coli*, the gene organization and regulation is similar to that in *P. putida* and not to that of the opportunistic human pathogen *P. aeruginosa*.

4. LYSINE BIOSYNTHESIS

Two different routes for lysine biosynthesis have been described in bacteria, the diaminopimelic pathway and the α -amino adipate pathway. The second was initially identified in the hyperthermophilic bacterium

*Thermus thermophilus*¹⁷. Genes homologous to those of the aminoadipate pathway have so far been only identified in *Deinococcus radiodurans* and in the archaea *Pyrococcus horikoshii* and *Pyrococcus abyssi*^{32, 33}. Thus, the diaminopimelic pathway seems to be widespread among prokaryotes⁵¹, and it is the one that appears to be used by *Pseudomonas* for lysine biosynthesis. It is a key anabolic route, leading to the synthesis of the amino acids lysine, methionine and threonine (which is also a precursor for isoleucine synthesis), and to meso-diaminopimelic acid, an essential component of the cell wall of Gram-negative bacteria. This route is also implicated in the synthesis of tabtoxin, a phytotoxic and antimicrobial compound produced by *P. syringae*²⁰, and of an intracellular iron chelator²³, both derived from one of the intermediaries in the route, tetrahydrodipicolinate.

The precursor of this pathway is aspartic acid, which is first phosphorylated by aspartate kinase, the product of the *lysC* gene (although the existence of several enzymes catalyzing the ATP-dependent phosphorylation of aspartate is common among various microorganisms). The second step is catalyzed by a key enzyme in the pathway, aspartate- β -semialdehyde dehydrogenase, encoded by the *asd* gene¹³. Mutations in this gene give rise to lysine, methionine and threonine auxotrophies, and cause a strict requirement for exogenous addition of diaminopimelic acid, necessary for the production of peptidoglycan. This characteristic has been exploited to design biological containment systems for genetically modified microorganisms⁴¹ and for in vivo expression technology (see related chapter in Volume 1). Aspartate- β -semialdehyde dehydrogenase of *P. aeruginosa* has been recently purified and its kinetic parameters appear to be very similar to those of its *E. coli* counterpart³⁰. Aspartate- β -semialdehyde is conjugated with pyruvate by DapA (dihydrodipicolinate synthase) and the product is further reduced to tetrahydrodipicolinate by DapB²⁰. Three different routes have been proposed to branch out from this compound, although only one is generally present in a single organism. Tetrahydrodipicolinate can be directly converted into meso-diaminopimelic acid by diaminopimelate dehydrogenase, or either acylated or succinylated⁵¹. Although the available information is incomplete and somewhat scattered, it appears that the pathway followed by *Pseudomonas* corresponds to the succinate branch (Figure 3). In this route, the aminotransferase enzyme responsible for the incorporation of the amino group to *N*-succinyl ketopymelate (DapC) has been an enigma for a long time. A gene encoding *N*-succinyl diaminopimelate aminotransferase (*dapC*) has been recently identified in *Bordetella pertussis*⁹. However, it appears that in *E. coli* this activity is carried out by the product of *argD*, *N*-acetylornithine aminotransferase, an enzyme in the arginine metabolic pathway¹⁸. It is unclear how this step is carried out in *Pseudomonas*. A gene annotated as *dapC* can be found in the genome of *P. aeruginosa*, coding for a protein 67% identical to DapC of *B. pertussis*. Similar proteins are also present

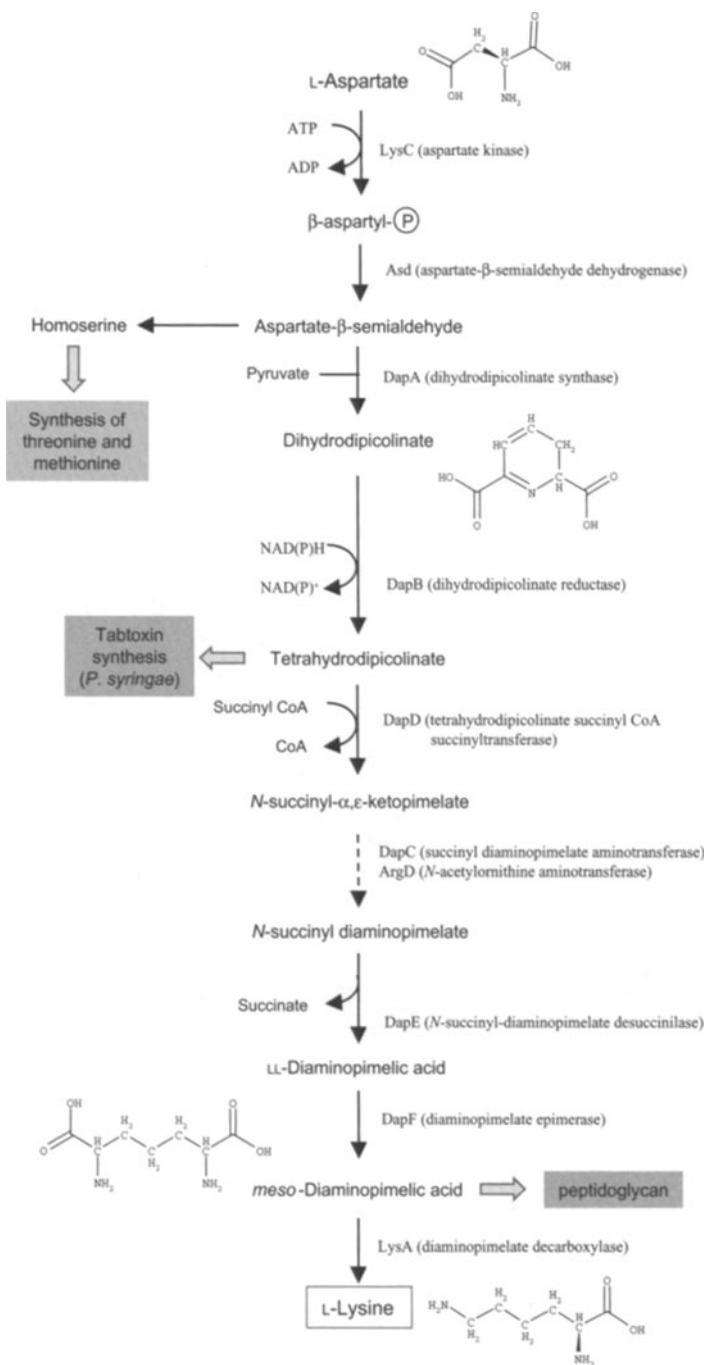


Figure 3. Predicted lysine biosynthetic pathway in *Pseudomonas*. A broken arrow indicates a step not fully characterized.

in other sequenced *Pseudomonas*, and thus, although their actual role remains to be elucidated, it seems probable that these proteins carry out the mentioned transamination reaction.

N-succinyl diaminopimelate is converted into LL-diaminopimelate after removal of the succinyl group by DapE (*N*-succinyl diaminopimelate desuccinylase)¹. An epimerase, encoded by *dapF* is then responsible for the conversion of LL-diaminopimelate into DL (*meso*)-diaminopimelate, which will be incorporated in the synthesis of cell wall or act as the direct precursor of lysine. This last step of the pathway is carried out by diaminopimelate decarboxylase, a constitutively expressed, pyridoxal phosphate-dependent enzyme²⁴ encoded by *lysA*, which in the case of *P. aeruginosa* is closely related to diaminopimelate decarboxylases of Gram-positive organisms²⁵. This decarboxylase, as well as other enzymes in the route (namely LysC, Asd and DapE), are also evolutionarily related to enzymes in the arginine biosynthetic pathway⁵¹.

Homologues of *lysA*, *dapA*, *dapB*, *asd* and *dapE* are present in all sequenced *Pseudomonas* species, and at least two putative aspartate kinase genes can be found in the *P. putida* KT2440 genome, one of them being 90% identical to LysC of *P. aeruginosa*. Interestingly, in *P. putida* two homologues of *dapF*, encoding diaminopimelate epimerase can also be found, one closely related to *dapF* of other *Pseudomonas* and the other showing similarities to *dapF* of *Nostoc* and some Gram-positive bacteria. As in proline biosynthesis, there are also two homologues of the last gene of the pathway, *lysA*, although this duplicity is not exclusive of KT2440 but appears also in *P. syringae*. Again, of the two proteins one is very similar to diaminopimelate decarboxylases of other *Pseudomonas*, while the other is related to equivalent enzymes of Enterobacteriaceae. Intriguingly, in all the sequenced *Pseudomonas* species, there is a second gene encoding a protein closely related to aspartate- β -semialdehyde dehydrogenase. In *P. aeruginosa*, this gene is located adjacent to the actual *asd* gene. However, this gene is either not functional, or its product has a different activity, since *asd* mutants of *P. aeruginosa* and *P. putida* show all of the previously mentioned auxotrophies.

5. LYSINE CATABOLISM

Many *Pseudomonas* strains can utilize L-lysine as a carbon and nitrogen source^{3, 6, 29}, although some strains of *P. aeruginosa* grow poorly on this amino acid as the sole carbon source⁷. However, derivatives that grow rapidly on lysine arise spontaneously during growth on the amino acid. D-lysine can also be catabolized, although this capability is limited, and in some cases it is harbored in a plasmid². The routes for lysine utilization by *P. aeruginosa* and

P. putida were characterized at the biochemical level in the 1970s, but only very recently the genetics of this pathway have begun to be explored in detail⁶ (O. Revelles *et al.*, unpublished). The routes deduced from biochemical and physiological studies, as well as from the few genetic data available are shown in Figure 4 and Table 1.

The OprD porin facilitates D- and L-lysine transport across the outer membrane⁴⁹. L-lysine then enters the cell by different possible mechanisms: a specific lysine/H⁺ symporter (LysP, characterized in *E. coli*; ref [46]) or a lysine/arginine permease. In *P. aeruginosa*, proteins similar to both systems can be found, a LysP homologue and ArcD, an arginine/ornithine antiporter which can also act as a lysine/ornithine exchanger⁵². This antiporter is also found in other *Pseudomonas*, which in *P. putida* is consistent with observations suggesting an overlap between lysine and arginine transport systems²⁹.

The first step in lysine utilization is a decarboxylation, which can occur through two different mechanisms. In the first one (the cadaverine pathway), L-lysine is transformed into cadaverine by lysine decarboxylase, a pyridoxal phosphate-dependent enzyme. Cadaverine deamination by cadaverine aminotransferase leads to a cyclic compound, 1-piperideine, which is converted into δ -aminovaleric acid by the action of piperideine dehydrogenase. In the alternative route (the monooxygenase pathway), L-lysine 2-monooxygenase is responsible for the decarboxylation of lysine to render δ -aminovaleramide, which will be deaminated by δ -aminovaleramide amidohydrolase to δ -aminovaleric acid⁴⁰. Thus, both the cadaverine and the monooxygenase pathways converge in the

Table 1. Enzymatic activities and lysine catabolic pathways in different *Pseudomonas* strains.

Organism	Lysine decarbox.	Lysine monoox.	L-Lysine-6-amino transf.	Lysine racem.	Main catabolic pathway
<i>P. fluorescens</i> ^{1, 2}	++	+++	+-	+	Cadaverine/monooxygenase/racemase
<i>P. putida</i> KT2440	-	+++	?	?	Monooxygenase
<i>P. putida</i> ATCC12633 ¹	-	++	+-	?	Monooxygenase
<i>P. putida</i> ATCC17472 ¹	-	++	+	?	Monooxygenase/aminotransferase
<i>P. aeruginosa</i> PAC1 ¹	++	-	+	?	Cadaverine
<i>P. multivorans</i> ¹	+-	-	+	?	Amino transferase/racemase?
<i>P. syringae</i>	(-)	?	?	?	Undetermined

¹Data from ref. [7].

²Data from ref. [8]. +: activity present; +-: present but minority; -: absent; (-): no homologue of the gene is present, but the activity has not been tested; ?: not determined.

same molecule, δ -aminovalerate. Although both routes exist in *Pseudomonas*, there seems to be a preference for one or the other in different species (Table 1). In *P. aeruginosa*, the cadaverine pathway constitutes the almost exclusive route, and no lysine monooxygenase activity can be detected⁷, whereas in *P. putida* lysine appears to be degraded preferentially via the monooxygenase pathway^{7, 29} (O. Revelles *et al.*, unpublished). In fact, cadaverine is used more efficiently than lysine as a carbon source by *P. aeruginosa*, given that, at least some strains appear to show low expression of lysine decarboxylase^{7, 38}. In contrast, cadaverine is toxic to *P. putida* KT2440 at concentrations over 5 mM (O. Revelles *et al.*, unpublished). In the case of *P. fluorescens*, both pathways appear to be present, without an obvious preference for one or the other^{7, 8}.

Aminovaleric acid is further metabolized by action of the enzyme δ -aminovalerate aminotransferase, which is encoded in *P. putida* by the *davT* gene. This reaction involves a transamination with α -ketoglutaric acid, and renders glutaric acid semialdehyde, which is in turn converted into glutaric acid by a dehydrogenase, encoded by *davD*. The absence of alternative routes for aminovalerate metabolism, at least in *P. putida*, is supported by the fact that a *davT* mutant is unable to utilize aminovaleric acid as a carbon or nitrogen source⁶. The genes coding for δ -aminovalerate aminotransferase and glutaric semialdehyde dehydrogenase, *davT* and *davD*, have so far been characterized only in *P. putida*⁶ where they form an operon (O. Revelles *et al.*, unpublished). Similar genes are found in other *Pseudomonas* species. However, the similarity of these genes to *gabT* and *gabD* (coding for γ -aminobutyrate [GABA] aminotransferase and succinate semialdehyde dehydrogenase, respectively, two enzymes in the GABA degradation pathway) make it difficult to assign them function without proper experimental evidences. Despite these similarities, δ -aminovalerate aminotransferase appears to be very specific for δ -aminovaleric acid¹⁴.

The rest of the genes in either the monooxygenase or the cadaverine pathway remain to be identified. The exceptions are a putative aminovaleramidase gene of *P. putida* (O. Revelles *et al.*, unpublished) and the lysine decarboxylase gene (called *cadA* in *E. coli*) for which a homologue is present in the genome of *P. fluorescens*. Two possible homologues also exist in *P. aeruginosa* (annotated as probable ornithine/arginine/lysine decarboxylases), although their similarity with known lysine decarboxylases is limited. It could be that the main physiological role of these putative decarboxylases is not in lysine degradation, which might explain why lysine is not as readily metabolizable as cadaverine by *P. aeruginosa*. No *cadA* homologue can be found in *P. syringae*, nor in *P. putida*, consistently with the prevalence of the monooxygenase pathway in this last organism.

Two other possibilities for L-lysine catabolism have been described (Table 1). One is through the action of an aminotransferase (lysine-6-aminotransferase) acting directly on L-lysine, to render piperidine-6-carboxylate, a cyclic intermediary in the catabolism of D-lysine⁷. D-lysine can be utilized by

Pseudomonas, either directly or after isomerization of L-lysine, via piperideine-2-carboxylate, pipecolate, piperideine-6-carboxylate and L-aminoadipate (Figure 4), rendering L-glutamic acid^{2, 37, 38}. However, both the isomerization and the lysine-6-aminotransferase activity, if present, are secondary. *P. putida* mutants blocked in the route for catabolism of L-lysine are unable to grow on this L-amino acid, despite the existence of a racemase activity, indicating that this activity is insufficient, and that D-lysine is preferentially utilized through an independent pathway³⁷ and not through isomerization. In *Pseudomonas oleovorans*, the racemase and the D-lysine route are plasmid-encoded². When the bacterium is cured of this so-called OCT plasmid, it is no longer able to grow on D-lysine, and also shows reduced transport of the amino acid into the cell, indicating that besides the enzymatic activities, a specific D-lysine transporter is also plasmid-encoded². However, none of the D-lysine utilization genes present in this plasmid has been characterized.

Little is known about the regulatory mechanisms controlling the lysine catabolic pathways. Some enzymatic activities are induced by lysine, and lysine transport is activated by lysine and pipecolate^{3, 29, 37}. The first three enzymes in the cadaverine route of *P. aeruginosa* are also induced during growth on cadaverine^{7, 14}. Despite the relative independence between the D- and the L-lysine pathways, there appears to be cross-induction of some enzymatic activities in one pathway by intermediates of the other³⁷. In *P. putida*, expression of the *davDT* operon is increased in the presence of lysine or δ -aminovalerate, which appears to be the true inductor⁶. As with the proline catabolic genes, expression of *davD* and *davT* is also activated by root exudates, both in vitro and in the rhizosphere. Further analysis of the regulatory circuits in lysine catabolism awaits the molecular genetic characterization of this complex pathway.

6. PROLINE AND LYSINE METABOLISM GENES IN SEQUENCED *PSEUDOMONAS*

The genomic data obtained after the completion of the sequencing projects of *Pseudomonas* strains can provide important clues to link enzymatic activities in different metabolic pathways with their corresponding genes, by comparison with the data available from other microorganisms. Table 2 summarizes our current knowledge of the genetics of proline and lysine metabolism in *Pseudomonas*. A comparative list of genes is compiled from *P. aeruginosa*, *P. putida* and *P. syringae*, along with their functions and the corresponding locus accession number in each of these three organisms, whose genomes are sequenced and fully annotated. The list includes previously characterized genes, genes annotated in the databases and others identified by

Table 2. Genomic survey of functions involved in proline and lysine metabolism in sequenced *Pseudomonas*.

Gene name	Protein function	Locus ^a		
		PAO1	KT2440	DC3000
<i>Proline synthesis</i>				
<i>proA</i>	γ-glutamyl phosphate reductase	PA4007	PP4811	PSPTO4829
<i>proB</i>	Glutamate kinase	PA4565	PP0691	PSPTO0800
<i>proC</i>	Δ ¹ -pyrroline-5-carboxylate reductase	PA0393	PP5095 (PP3778)	PSPTO5047
<i>ocd</i>	Putative ornithine cyclodeaminase	(PA4908)	PP3533	—
<i>Lysine synthesis</i>				
<i>lysC</i>	Aspartate kinase	PA0904	PP4473	PSPTO1843
<i>asd</i>	Aspartate-β-semialdehyde dehydrogenase	PA3117 (PA3116)	PP1989 (PP1992)	PSPTO2176 (PSPTO3819)
<i>dapA</i>	Dihydrodipicolinate synthase	PA1010	PP1237	PSPTO3953
<i>dapB</i>	Dihydrodipicolinate reductase	PA4759	PP4725	PSPTO4503
<i>dapC</i>	N-succinyl diaminopimelate aminotransferase	PA3659	PP1588	PSPTO1531
<i>dapD</i>	Tetrahydrodipicolinate succinylase	PA3666	PP1530	PSPTO1528
<i>dapE</i>	Succinyl-diaminopimelate desuccinylase	PA1162	PP1525	PSPTO1523
<i>dapF</i>	Diaminopimelate epimerase	PA5278	PP5228 (PP3790)	PSPTO0224
<i>lysA</i>	Diaminopimelate decarboxylase	PA5277	PP5227 (PP0277)	PSPTO0225 (PSPTO0209)
<i>Proline catabolism</i>				
<i>putA</i>	Proline dehydrogenase, Δ ¹ -pyrroline-5-carboxylate dehydrogenase (and regulatory ^d)	PA0782	PP4947	PSPTO5017
<i>Lysine catabolism</i>				
<i>cadA</i>	Lysine decarboxylase	(PA1818) (PA1346)	—	—

Table 2. Continued

Gene name	Protein function	Locus ^a		
		PAO1	KT2440	DC3000
<i>davT^b</i>	δ-aminovalerate aminotransferase	PA0266	PP0214	PSPTO0301
<i>davD^c</i>	Glutaric semialdehyde dehydrogenase	PA0265	PP0213	PSPTO0300
<i>Transport</i>				
<i>putP</i>	Proline/Na ⁺ symporter	PA0783	PP4946	PSPTO5016
<i>proP</i>	Proline permease, MFS family	(PA4343)	PP2914	(PSPTO3801)
<i>proV</i>	ProU ABC transporter, ATP-binding protein	PA5376	PP0294	PSPTO0462
		PA5094	PP2774 ^e	PSPTO3060
<i>proW</i>	ProU ABC transporter, membrane protein	PA5377	PP0295	PSPTO0463
		PA5095	PP2774 ^e	PSPTO3059
<i>proX</i>	ProU ABC transporter, periplasmic protein	PA5378	PP0296	PSPTO0464
		PA5096	PP2775	PSPTO3058
<i>proY</i>	Proline permease (cryptic? ^f)	PA5097	PP5031	PSPTO5276
<i>lysP</i>	Lysine permease	PA4628	(PP3727)	(PSPTO5356)
<i>acrD</i>	Arginine/ornithine, lysine/ornithine antiporter	PA5170	PP1002	(PSPTO2026)
<i>oprD</i>	Outer membrane porin	PA0958	PP1206	PSPTO3987
<i>Regulation</i>				
<i>oruR</i>	Ornithine utilization regulator	PA0831	(PP5339)	(PSPTO3805)
<i>chrA</i>	Two-component system, sensory box histidine kinase; amino acid utilization regulator	PA4725	PP4695	PSPTO0965
<i>chrB</i>	Two-component system, response regulator	PA4726	PP4696	PSPTO0964
<i>pruR</i>	Proline catabolism regulator	PA0780	(PP4511)	(PSPTO1213)

^aLoci corresponding to each gene in *P. aeruginosa* PAO1, *P. putida* KT2440 and *P. syringae* DC3000, identified either by annotation (from www.tigr.org and www.pseudomonas.com) or by sequence similarity. Loci in brackets show low similarity with the characterized genes, so their actual function is unclear. ^b*davT* and *davD* are annotated as *gabT* (γ-aminobutyrate aminotransferase) and *gabD* (succinate semialdehyde dehydrogenase), but their role in lysine catabolism has been established experimentally in *P. putida*. ^cExcept in PAO1. ^ePP2774 encodes a fusion protein. ^f*proY* is cryptic in *Salmonella*.

sequence similarity searches with proteins that have been characterized in other organisms. These comparative studies have two important facets. First, they provide a genetic basis for biochemical observations, such as the already discussed absence of a *cadA* homologue in *P. putida*. These data may also give important clues and direct future research in amino acid transport and metabolism, such as, for example, investigating the actual role of the putative *dapC* gene found in all three strains, or of the homologues of the cryptic ProY transporter in proline acquisition.

REFERENCES

1. Bouvier, J., Richaud, C., Higgins, W., Bogler, O., and Stragier, P., 1992, Cloning, characterization, and expression of the *dapE* gene of *Escherichia coli*. *J. Bacteriol.*, 174:5265–5271.
2. Cao, X., Kolonay J., Jr, Saxton, K.A., and Hartline, R.A., 1993, The OCT plasmid encodes D-lysine membrane transport and catabolic enzymes in *Pseudomonas putida*. *Plasmid*, 30:83–89.
3. Chang, Y.F. and Adams, E., 1977, Factors influencing growth on L-lysine by *Pseudomonas*. Regulation of terminal enzymes in the delta-aminovalerate pathway and growth stimulation by alpha ketoglutarate. *J. Biol. Chem.*, 252:7987–7991.
4. Deutch, A.H., Rushlow, K.E., and Smith, C.J., 1984, Analysis of the *Escherichia coli* *proBA* locus by DNA and protein sequencing. *Nucleic Acids Res.*, 12:6337–6355.
5. Ekena, K. and Maloy, S., 1990, Regulation of proline utilization in *Salmonella typhimurium*: How do cells avoid a futile cycle? *Mol. Gen. Genet.*, 220:492–494.
6. Espinosa-Urgel, M. and Ramos, J.L., 2001, A *Pseudomonas putida* aminotransferase involved in lysine catabolism is induced in the rhizosphere. *Appl. Environ. Microbiol.*, 67:5219–5224.
7. Fothergill, J.C. and Guest, J.R., 1977, Catabolism of L-lysine by *Pseudomonas aeruginosa*. *J. Gen. Microbiol.*, 99:139–155.
8. Friede, J.D. and Henderson, L.M., 1976, Metabolism of 5-hydroxylysine in *Pseudomonas fluorescens*. *J. Bacteriol.*, 127:1239–1247.
9. Fuchs, T.M., Schneider, B., Krumbach, K., Eggeling, L., and Gross R., 2000, Characterization of a *Bordetella pertussis* diaminopimelate (DAP) biosynthesis locus identifies *dapC*, a novel gene coding for N-succinyl-L, L-DAP aminotransferase. *J. Bacteriol.*, 182:3626–3631.
10. Glass, N.L. and Kosuge, T., 1988, Role of indoleacetic acid-lysine synthetase in regulation of indoleacetic acid pool size and virulence of *Pseudomonas syringae* subsp. savastanoi. *J. Bacteriol.*, 170:2367–2373.
11. Hayzer, D.J. and Leisinger, T., 1980, The gene-enzyme relationships of proline biosynthesis in *Escherichia coli*. *J. Gen. Microbiol.*, 118:287–293.
12. Hebert, M.D. and Houghton, J.E., 1997, Regulation of ornithine utilization in *Pseudomonas aeruginosa* (PAO1) is mediated by a transcriptional regulator, OruR. *J. Bacteriol.*, 179:7834–7842.
13. Hoang, T.T., Williams, S., Schweizer, H.P., and Lam, J.S., 1997, Molecular genetic analysis of the region containing the essential *Pseudomonas aeruginosa* *asd* gene encoding β -aspartate semialdehyde dehydrogenase. *Microbiology*, 143:899–907.
14. Ichihara, A., Ichihara, E.A., and Suda, M., 1960, Metabolism of L-lysine by bacterial enzymes IV. δ -aminovaleric acid-glutamic acid transaminase. *J. Biochem.*, 48:412–420.
15. Jovanovich, S.B., Martinell, M., Record M.T., Jr, and Burgess, R.R., 1988, Rapid response to osmotic upshift by osmoregulated genes in *Escherichia coli* and *Salmonella typhimurium*. *J. Bacteriol.*, 170:534–539.

16. Kenklies, J., Ziehn, R., Fritsche, K., Pich, A., and Andreessen, J.R., 1999, Proline biosynthesis from L-ornithine in *Clostridium sticklandii*: Purification of delta1-pyrroline-5-carboxylate reductase, and sequence and expression of the encoding gene, *proC*. *Microbiology*, 145:819–826.
17. Kosuge, T. and Hoshino, T., 1998, Lysine is synthesized through the alpha-aminoadipate pathway in *Thermus thermophilus*. *FEMS Microbiol. Lett.*, 169:361–367.
18. Ledwidge, R. and Blanchard, J.S., 1999, The dual biosynthetic capability of N-acetylornithine aminotransferase in arginine and lysine biosynthesis. *Biochemistry*, 38:3019–3024.
19. Liao, M.K., Gort, S., and Maloy, S., 1997, A cryptic proline permease in *Salmonella typhimurium*. *Microbiology*, 143:2903–2911.
20. Liu, L., and Shaw, P.D., 1997, Characterization of *dapB*, a gene required by *Pseudomonas syringae* pv. tabaci BR2.024 for lysine and tabtoxinine-beta-lactam biosynthesis. *J. Bacteriol.*, 179:507–513.
21. Mahan, M.J. and Csonka, L.N., 1983, Genetic analysis of the *proBA* genes of *Salmonella typhimurium*: Physical and genetic analyses of the cloned *proB*⁺*A*⁺ genes of *Escherichia coli* and of a mutant allele that confers proline overproduction and enhanced osmotolerance. *J. Bacteriol.*, 156:1249–1262.
22. Manna, D. and Gowrishankar, J., 1994, Evidence for involvement of proteins HU and RpoS in transcription of the osmoreponsive *proU* operon in *Escherichia coli*. *J. Bacteriol.*, 176:5378–5384.
23. Maringanti, S. and Imlay, J.A., 1999, An intracellular iron chelator pleiotropically suppresses enzymatic and growth defects of superoxide dismutase-deficient *Escherichia coli*. *J. Bacteriol.*, 181:3792–3802.
24. Martin, C., Cami, B., Borne, F., Jeenes, D.J., Haas, D., and Patte, J.C., 1986, Heterologous expression and regulation of the *lysA* genes of *Pseudomonas aeruginosa* and *Escherichia coli*. *Mol. Gen. Genet.*, 203:430–434.
25. Martin, C., Cami, B., Yeh, P., Stragier, P., Parsot, C., and Patte, J.C., 1988, *Pseudomonas aeruginosa* diaminopimelate decarboxylase: Evolutionary relationship with other amino acid decarboxylases. *Mol. Biol. Evol.*, 5:549–559.
26. Meile, L. and Leisinger, T., 1982, Purification and properties of the bifunctional proline dehydrogenase/1-pyrroline-5-carboxylate dehydrogenase from *Pseudomonas aeruginosa*. *Eur. J. Biochem.*, 129:67–75.
27. Meile, L., Soldati, L., and Leisinger, T., 1982, Regulation of proline catabolism in *Pseudomonas aeruginosa* PAO. *Arch. Microbiol.*, 132:189–193.
28. Mellies, J., Wise, A., and Villarejo, M., 1995, Two different *Escherichia coli proP* promoters respond to osmotic and growth phase signals. *J. Bacteriol.*, 177:144–151.
29. Miller, D.L. and Rodwell, V.W., 1971, Metabolism of basic amino acids in *Pseudomonas putida*. Properties of the inducible lysine transport system. *J. Biol. Chem.*, 246:1765–1771.
30. Moore, R.A., Bocik, W.E., and Viola, R.E., 2002, Expression and purification of aspartate beta-semialdehyde dehydrogenase from infectious microorganisms. *Protein Expr. Purif.*, 25:189–194.
31. Nakada, Y., Nishijyo, T., and Itoh, Y., 2002, Divergent structure and regulatory mechanism of proline catabolic systems: Characterization of the *putAP* proline catabolic operon of *Pseudomonas aeruginosa* PAO1 and its regulation by PruR, an AraC/XylS family protein. *J. Bacteriol.*, 184:5633–5640.
32. Nishida, H., 2001, Distribution of genes for lysine biosynthesis through the aminoadipate pathway among prokaryotic genomes. *Bioinformatics*, 17:189–191.
33. Nishida, H., Nishiyama, M., Kobashi, N., Kosuge, T., Hoshino, T., and Yamane, H., 1999, A prokaryotic gene cluster involved in synthesis of lysine through the amino adipate pathway: A key to the evolution of amino acid biosynthesis. *Genome Res.*, 9:1175–1183.

34. Nishijyo, T., Haas, D., and Itoh, Y., 2001, The CbrA-CbrB two-component regulatory system controls the utilization of multiple carbon and nitrogen sources in *Pseudomonas aeruginosa*. *Mol. Microbiol.*, 40:917–931.
35. Nowak-Thompson, B., Chaney, N., Wing, J.S., Gould, S.J., and Loper, J.E., 1999, Characterization of the pyoluteorin biosynthetic gene cluster of *Pseudomonas fluorescens* Pf-5. *J. Bacteriol.*, 181:2166–2174.
36. Omori, K., Suzuki, S., Imai, Y., and Komatsubara, S., 1991, Analysis of the *Serratia marcescens proBA* operon and feedback control of proline biosynthesis. *J. Gen. Microbiol.*, 137:509–517.
37. Payton, C.W. and Chang, Y.-F., 1982, Δ^1 -piperidine-2-carboxylate reductase of *Pseudomonas putida*. *J. Bacteriol.*, 149:864–871.
38. Rahman, M. and Clarke, P.H., 1980, Genes and enzymes of lysine catabolism in *Pseudomonas aeruginosa*. *J. Gen. Microbiol.*, 116:357–369.
39. Ramos, J.L., Gallegos, M.T., Marqués, S., Ramos-González, M.I., Espinosa-Urgel, M., and Segura, A., 2001, Responses of gram-negative bacteria to certain environmental stressors. *Curr. Opin. Microbiol.*, 4: 166–171.
40. Reitz, M.S. and Rodwell, V.W. 1970. δ -Aminovaleramidase of *Pseudomonas putida*. *J. Biol. Chem.* 245:3091–3096.
41. Ronchel, M.C. and Ramos, J.L., 2001, Dual system to reinforce biological containment of recombinant bacteria designed for rhizoremediation. *Appl. Environ. Microbiol.*, 67:2649–2656.
42. Samartzidou, H. and Delcour, A.H., 1999, Excretion of endogenous cadaverine leads to a decrease in porin-mediated outer membrane permeability. *J. Bacteriol.*, 181:791–798.
43. Samartzidou, H., Mehrazin, M., Xu, Z., Benedik, M.J., and Delcour, A.H., 2003, Cadaverine inhibition of porin plays a role in cell survival at acidic pH. *J. Bacteriol.*, 185:13–19.
44. Savioz, A., Jeenes, D.J., Kocher, H.P., and Haas, D., 1990, Comparison of *proC* and other housekeeping genes of *Pseudomonas aeruginosa* with their counterparts in *Escherichia coli*. *Gene*, 86:107–111.
45. Stalon, V., Vander Wauven, C., Momin, P., and Legrain, C., 1987, Catabolism of arginine, citrulline and ornithine by *Pseudomonas* and related bacteria. *J. Gen. Microbiol.*, 133:2487–2495.
46. Steffes, C., Ellis, J., Wu, J., and Rosen, B.P., 1992, The *lysP* gene encodes the lysine-specific permease. *J. Bacteriol.*, 174:3242–3249.
47. Thomas, M.G., Burkart, M.D., and Walsh, C.T., 2002, Conversion of L-proline to pyrrolyl-2-carboxyl-S-PCP during undecylprodigiosin and pyoluteorin biosynthesis. *Chem. Biol.*, 9:171–184.
48. Townsend, D.E., Kaenjak, A., Jayaswal, R.K., and Wilkinson, B.J., 1996, Proline is biosynthesized from arginine in *Staphylococcus aureus*. *Microbiology*, 142:1491–1497.
49. Trias, J. and Nikaido, H., 1990, Protein D2 channel of the *Pseudomonas aeruginosa* outer membrane has a binding site for basic amino acids and peptides. *J. Biol. Chem.*, 265:15680–15684.
50. Tricot, C., Stalon, V., and Legrain, C., 1991, Isolation and characterization of *Pseudomonas putida* mutants affected in arginine, ornithine and citrulline catabolism: Function of the arginine oxidase and arginine succinyltransferase pathways. *J. Gen. Microbiol.*, 137:2911–2918.
51. Velasco, A.M., Leguina, J.I., and Lazcano, A., 2002, Molecular evolution of the lysine biosynthetic pathways. *J. Mol. Evol.*, 55:445–459.
52. Verhoogt, H.J., Smit, H., Abee, T., Gamper, M., Driessen, A.J., Haas, D., and Konings, W.N., 1992, *arcD*, the first gene of the *arc* operon for anaerobic arginine catabolism in *Pseudomonas aeruginosa*, encodes an arginine–ornithine exchanger. *J. Bacteriol.*, 174:1568–1573.
53. Vilchez, S., 2000. PhD. thesis. Universidad de Granada, Spain.

54. Vilchez, S., Manzanera, M., and Ramos, J.L., 2000, Control of expression of divergent *Pseudomonas putida put* promoters for proline catabolism. *Appl. Environ. Microbiol.*, 66:5221–5225.
55. Vilchez, S., Molina, L., Ramos, C., and Ramos, J.L., 2000, Proline catabolism by *Pseudomonas putida*: Cloning, characterization, and expression of the *put* genes in the presence of root exudates. *J. Bacteriol.*, 182:91–99.

ACTIVATION OF TRANSCRIPTION INITIATION AND REGULATION OF TRYPTOPHAN BIOSYNTHESIS IN FLUORESCENT PSEUDOMONADS

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1. ORGANIZATION AND EXPRESSION OF *trp* GENES

1.1. Overview

Although the *Escherichia coli* *trp* operon is a favored textbook example of transcriptional regulation, the arrangement of *trp* genes and their pattern of regulation in *E. coli* is not at all typical^{27, 28, 48, 82}. Recall that in *E. coli*, the products of *five* contiguous genes, *trpE*, (*G*)*D*, *C(F)*, *B*, and *A*, are required for the conversion of chorismate to tryptophan (Figure 1). The operon is negatively regulated by TrpR (repressor) in response to tryptophan and by a transcription attenuator whose activity is modulated by acylated tRNA^{trp} (see refs [66], [67], [119]). The five genes in the operon actually encode *seven* enzymatic activities since the bifunctional *trp(G)D* and *trpC(F)* genes encode fusion proteins each of which has two independent enzymatic functions.

The arrangement of *trp* genes and the way they are regulated in the fluorescent *Pseudomonads* contrast sharply with the pattern observed in *E. coli* in several important ways. First, and most obvious, in the fluorescent

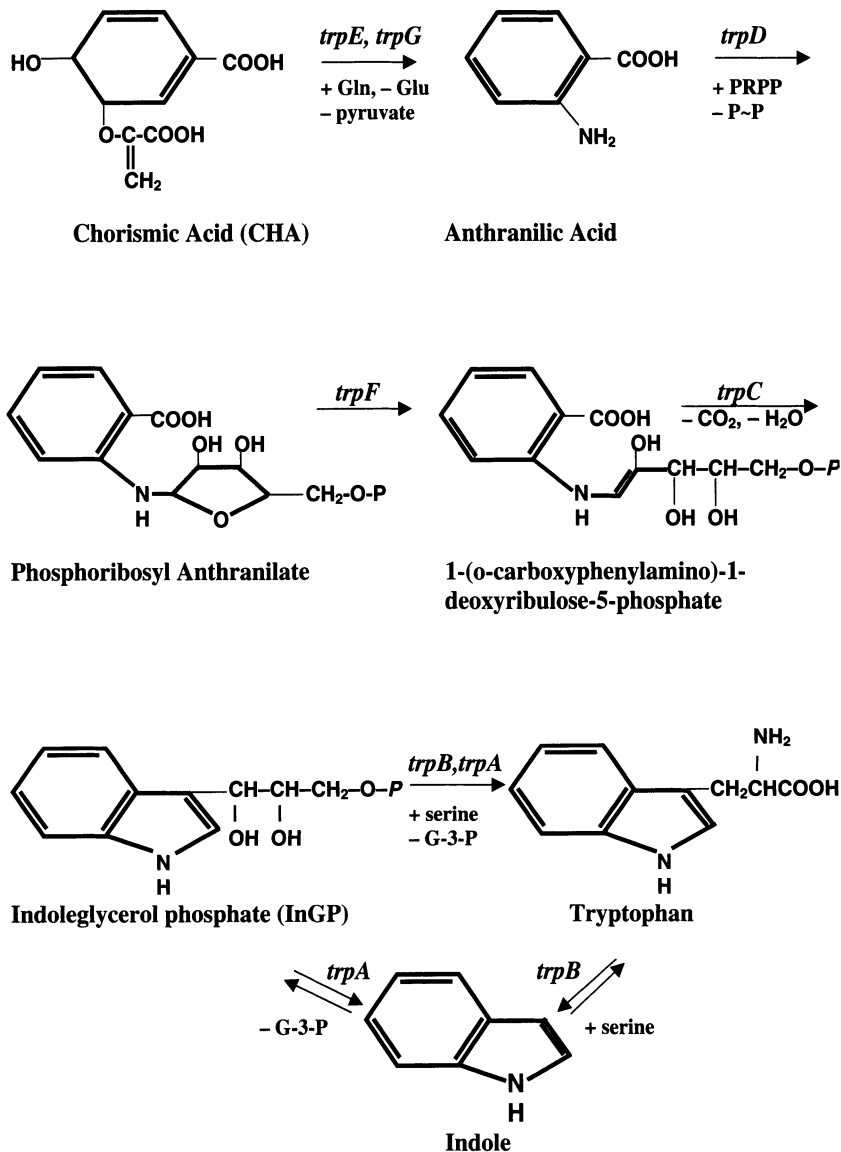


Figure 1. Biosynthesis of tryptophan. The steps from chorismate to tryptophan and genes whose products catalyze each step are indicated. Enzymatic reactions are catalyzed sequentially by anthranilate synthase (AS), which acts as an oligomer containing subunits specified by *trpE* and *trpG*; phosphoribosyl transferase (PRI); phosphoribosyl anthranilate isomerase (PRAI); indoleglycerol phosphate synthase (IGPS); and tryptophan synthase B and A. The subunits of tryptophan synthase function as an oligomer, but can carry out the two steps required for the synthesis of tryptophan from InGP and serine separately. For detailed descriptions of these enzymes, see refs [27], [28], [82].

Pseudomonads, the *trp* genes are not encoded in a single messenger RNA molecule, but instead comprise four discrete transcriptional units encoding *trpE*, *trpGDC*, *trpBA*, and *trpF*. Each gene encodes a monofunctional polypeptide chain. Second, since there is no TrpR homolog in fluorescent Pseudomonads, none of the transcription units is regulated by a repressor. The primary, and perhaps only, mechanism of tryptophan-mediated regulation of *trpE* and *trpGDC* transcription is attenuation⁸⁷. Furthermore, in at least *Pseudomonas putida*, *Pseudomonas syringae*, and *Pseudomonas aeruginosa*, *trpB* and *trpA*, the genes that encode the subunits of tryptophan synthase, constitute a single operon that is regulated positively by the product of the *trpI* gene^{5, 19, 21}. Activation of *trpBA* also requires indoleglycerol phosphate (InGP), a pathway intermediate (Figure 1) and substrate for tryptophan synthase⁷¹. Thus, the *trpBA* operon is regulated only indirectly by tryptophan, primarily through feedback inhibition of the first enzyme in the pathway, anthranilate synthase, which thereby reduces the synthesis of InGP (see below). Finally, *trpF* expression is not regulated by tryptophan, even in *trp* auxotrophs^{17, 29, 74, 88}.

While the details of tryptophan biosynthesis have been well understood for several decades, the regulatory mechanisms involved still demand attention, both because of their importance in understanding gene function and because they have had a profound influence on attempts to understand how gene rearrangements fuel evolutionary change in biosynthetic pathways^{15, 28, 30, 56, 82, 92}. TrpI, a member of the LysR family of transcriptional regulators^{21, 45, 104}, was found initially only in *P. putida*, *P. aeruginosa*, and *P. syringae*^{5, 19, 21, 71}. However, a survey of the NCBI database as of May 1, 2003 revealed *trpI* homologs in the genomes of *Pseudomonas fluorescens*, *Xanthomonas axonopodis*, *Xanthomonas campestris*, and *Azotobacter vinelandii*. These (and other) comparative genomic data may ultimately contribute to a clearer understanding at the biochemical level of phylogenetic relationships among the fluorescent Pseudomonads and closely related proteobacteria.

This chapter will focus primarily on tryptophan biosynthesis in fluorescent Pseudomonads or in related species that provide relevant comparisons to the pathway in fluorescent Pseudomonads. In addition, the chapter includes new information—obtained from the genome database—that is especially pertinent to understanding the regulation of the pathway. Several reviews of the structure and function of *trp* enzymes have been published previously^{77, 82, 91, 122, 123}.

1.2. Map Location of *trp* Genes

It has been known for some time that the map locations of specific genes in several pathways vary significantly, even among related species^{28, 48}. Table 1 compares distances in base pairs (bp) of each of the *trp* gene clusters from the replication origin (actually from the *dnaA* gene) for several species of

Table 1. Chromosomal locations of *trp* genes.

Species	Genome size (M)	Distance from <i>dnaA</i> (million bp) ^a				
		<i>trpBA</i>	<i>trpE</i>	<i>trpGDC</i>	<i>trpF</i>	<i>rpoD</i>
<i>P. aeruginosa</i>	6.2	0.038	0.671	0.705	3.5	0.635
<i>P. putida</i>	6.2	0.092	0.506	0.510	2.26	0.589
<i>P. syringae</i>	6.4	0.179	0.624	0.653	4.33	0.471
<i>X. campestris</i>	5.1	3.02	0.559	0.570	3.02	4.4
<i>X. axonopodis</i>	5.2	3.17	0.566	0.570	3.17	4.5
<i>R. solanacearum</i>	3.7	1.54	0.60	0.60	1.54	1.3

^aData were taken from the NCBI genome data base as of May 30, 2003.

Pseudomonads. While the positions of *trpBA*, *trpE*, and *trpGDC* in the fluorescent species (homology group I of Palleroni *et al.*⁹⁰) are relatively constant in relation to the overall size of the genome, the position of *trpF* varies substantially. Distances among the three most closely linked operons, and especially between *trpE* and *trpGDC*, could easily change due to insertions or deletions, integration of prophages, or insertion of transposable elements. More substantial differences may involve more dramatic chromosome rearrangements.

In the two *Xanthomonas* species (homology group V of Palleroni *et al.*⁹⁰), the positions of *trpE* and *trpGDC* are fairly close to the positions of the corresponding genes in group I species, but the position of *trpBA* is very different; furthermore, *trpBA* and *trpF* are very tightly linked, separated only by *trpI*. In addition, in these two species, the position of *rpoD*, which was arbitrarily chosen as an additional reference point, is dramatically different from its position in the fluorescent *Pseudomonads*. Finally, in *Ralstonia solanacearum* (formerly *Pseudomonas solanacearum*), a member of homology group II⁹⁰, all the *trp* genes are located within about 1 million bp of each other. This is similar, but not as extreme as the clustering of the corresponding genes in *Burkholderia cepacia* (formerly *Pseudomonas cepacia*), also a member of homology group II. Although the gene order in the two species appears to be the same, and in both cases the *trpABF* cluster is linked to *leu*, all the *trp* genes in *B. cepacia* are located within a single 28 kb segment⁷³.

1.3. Regulation by Tryptophan

The response of *trp* genes to exogenous tryptophan in vivo is usually much greater in *trp* auxotrophs than in *trp* prototrophs, and is greater in enteric bacteria than in many *Pseudomonads* (Table 2). In *E. coli trp* auxotrophs grown in minimal medium, tryptophan decreases *trp* gene expression by a factor of 50–100, compared with a factor of 5–10 in prototrophs. (Prototrophs

Table 2. *Trp* gene organization and regulation by tryptophan.

Species	Gene organization ^a	Degree of repression ^b			
		<i>trpE</i>	<i>trpDC</i>	<i>trpF</i>	<i>trpBA</i>
<i>E. coli</i> wt	<u>E-(G•D)-(C•F)-B-A</u>	5–10	5–10	n.a.	5–10
<i>trp</i> aux.		50–100	50–100	n.a.	50–100
<i>P. putida</i> wt	<u>E G-D-C F B-A</u>	~1.5	~1	~1	~7
<i>trp</i> aux.		5–20	3–6	~1.4	6–20 ^c
<i>P. aerug.</i> wt	<u>E G-D-C F B-A</u>	~1	~1	~1	8–12
<i>trp</i> aux.		3–17	10–20	1–1.5	~200 ^c
<i>C. acidovor.</i> ^d wt	<u>E G-D-C F- B-A</u>	~1	~1	~1	~1
<i>trp</i> aux.		~10	6–40	~1.2	~1–4
<i>A. calc.</i> ^e wt	<u>E G-D-C F-B A</u>	~1	~1	~1.8	~1
<i>trp</i> aux.		~1.6	~3–4	~3	~1.6

^aOperons are underlined; fused genes are denoted by bullets (•).

^bEnzyme activity in cells grown in the absence (or very low levels) of exogenous *trp* relative to activity in cells grown in the presence of excess *trp*.

^cNo induction is seen for auxotrophs unable to synthesize InGP; the auxotroph yielding high *trpBA* expression was *trpA*[−] for *P. aeruginosa* and *trpB*[−] for *P. putidash*.

^dFormerly called *Pseudomonas acidovorans*.

^ePrecise data for enzyme levels in *C. acidovorans trp* prototrophs were not presented in the indicated reference.

Abbreviations: n.a., assayed; wt, wild-type; aux., auxotroph.

References: *E. coli*^{55, 120}; *P. putida*²⁹; *P. aeruginosa*¹⁷; *C. acidovorans*¹³; *A. calcoaceticus*^{25, 104}.

are not fully derepressed because of the presence of endogenous tryptophan.) Significantly, in cells grown in minimal medium, there is little or no relief of attenuation unless specific steps are taken to completely deplete cells of endogenous tryptophan¹²⁰. Thus, the data in Table 2 reflect the TrpR-mediated effect of tryptophan. Relief of attenuation would increase enzyme levels by an additional factor of 6–7.

Data for several other species (Table 2) contrast sharply with the data for *E. coli*. Enzyme levels in prototrophs are hardly affected by tryptophan; this might be expected if the mechanism of regulation of the *trpE* and *trpGDC* operons were attenuation, since, as mentioned previously, conditions of extreme starvation are necessary for complete relief of attenuation in *E. coli*.

The data in Table 2 also provide the first clues for a different mode of regulation of *trpBA*. In fluorescent Pseudomonads, which encode *trpI*, expression of *trpBA* is increased substantially by tryptophan limitation in prototrophs, but this is not the case in a close relative, *Acinetobacter calcoaceticus*²⁵, or in *Comamonas acidovorans* (formerly *Pseudomonas acidovorans*)¹³. (Note that in *C. acidovorans*, a member of homology group III, *trpB* and *trpA* are separated by at least 250 bp⁶².) Furthermore, the level of *trpBA* expression is increased by exogenous tryptophan in a *trpA* or *trpB* auxotroph, but not in *trpE*, *D*, *C*, or *F* mutants, which are unable to synthesize InGP. Presumably, the

very high level of tryptophan synthase B in a *P. aeruginosa* *trpA* mutant¹⁷ is due to the accumulation of unusually high levels of InGP when cells are unable to convert InGP to indole (Figure 1). Finally, in *P. putida*²⁹, but not in *A. calcoaceticus*²⁵, the level of tryptophan synthase B can be induced approximately 200-fold in a *trpA-trpE* double mutant in the presence of low levels of tryptophan (necessary for cell growth) by the addition of anthranilate (which can be converted to InGP in vivo). A similar result—induction of *trpBA* by anthranilate—was obtained in a *P. aeruginosa* mutant blocked in the shikimic acid pathway, which is required for the production of chorismic acid (CHA) and thus for the synthesis of InGP (and tryptophan)¹⁷.

2. ACTIVATION OF *trpP_B*

2.1. General Activation Mechanisms

In general, DNA-bound activator proteins regulate prokaryotic transcription initiation through direct contact with RNAP^{11, 12}. This view is supported by: (a) isolation of mutant activators that bind DNA normally but fail to activate transcription^{47, 125}; (b) synergistic binding of a promoter-specific activator to its target site and RNAP to the promoter^{46, 52, 102}; (c) chemical cross-linking between an activator and the α subunit of RNAP²²; (d) defects in the response of RNAP to certain activators in vitro when 73 C-terminal amino acids of α are removed⁵³; and (e) activation-defective phenotypes of point mutations in the α gene (*rpoA*) in vitro and in vivo^{11, 12}. In these cases, the activator may facilitate the binding of the carboxy-terminal domain (CTD) of α to a suboptimal upstream recognition sequence located at about -50 to -60 relative to the transcription startsite. The prototype of the α -CTD recognition sequence is the UP element of the *rrnB* P1 promoter, which directly binds the isolated α subunit^{7, 103}. Activators may also contact other RNAP subunits. For example, λ cI protein activates the P_{RM} promoter by contacting the RNAP σ subunit^{64, 68, 84} and phage N4 single-stranded binding protein activates late promoters by interacting with the RNAP β' subunit⁷⁸.

At some promoters, DNA-bending induced by activator binding causes distortions in DNA structure that may result in activation independent of contact between the activator and RNAP^{3, 93, 96}. In these cases, the possibility of a direct activator–RNAP interaction was not ruled out, but there is strong evidence that a DNA distortion induced by bending is entirely responsible for activation of the *ilvP_G* promoter⁹³. The isolation of activator (CAP-cAMP) mutants that bend DNA normally but fail to activate transcription^{12, 125} suggests that bending in many cases may simply facilitate an RNAP–activator interaction. DNA bending proteins (such as the integration host factor, IHF)

may also facilitate DNA bending (or looping) required for the interaction of an upstream activator with RNAP^{24, 50}. For example, in *P. putida*, IHF-mediated DNA bending appears to be required to facilitate contact between XylR and RNAP at the σ^{54} -dependent TOL plasmid promoter, *Pu*⁹⁵. At this promoter, IHF also directly or indirectly affects the positioning of the α -CTDs in relation to a possible UP-like promoter element⁶⁹.

2.2. TrpI-Mediated Gene Activation

TrpI is a member of the widely dispersed LysR family of transcriptional regulatory proteins that have several common features^{45, 105}. They are about 300 amino acids in length and share extensive amino acid sequence homology, especially in the N-terminal 50–75 amino acids, which include the DNA binding (helix-turn-helix) domain; they usually function as activators of structural genes and repressors of their own synthesis; their activity is affected by a small inducer molecule, which usually is an intermediate in the pathway they regulate; they are encoded by genes that are transcribed divergently from their target operons. Several LysR proteins require the inducer to act at a step subsequent to initial DNA binding^{14, 36, 51, 109, 114}, perhaps by altering the conformation of the bound activator so that it can interact with RNAP.

2.2.1. Role of *TrpI*

Analysis of the effects of TrpI binding site mutations on activation *in vitro* led us to propose the following model (Model 1) for the activation of the *trpBA* promoter (*trpP_B*)³⁹: (a) A TrpI molecule, presumably a tetramer¹⁹, binds to site I, the stronger of two TrpI binding sites (Figure 2) to form *complex 1*. Binding to this site represses the *trpI* promoter (*trpP_I*), but is not sufficient to activate *trpP_B*. (b) A second TrpI molecule interacts with the first; this interaction is very strongly dependent on InGP^{19, 20}. (c) The second TrpI molecule establishes contacts with site II, which cannot bind TrpI in the

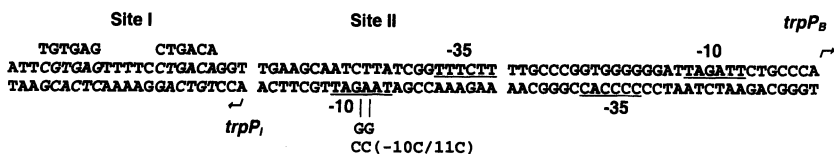


Figure 2. Nucleotide sequence of the *trpI-trpBA* control region of *P. aeruginosa* PAC174. Transcription startsites for *trpI* and *trpB* are indicated by arrows. Consensus -35 and -10 regions of *trpP_B* and *trpP_I* are underlined in the upper and lower strand, respectively. The presumed Trp I recognition sequence in site I is in italics and the consensus recognition sequence is indicated above the actual sequence (see refs [5], [21]). The site II mutation -10C/11C is in the -10 region of *trpP_I*.

absence of site I, to form *complex 2*. (d) TrpI bound to site II interacts with RNAP to stimulate transcription from *trpP_B*.

Since TrpI failed to stimulate transcription initiation in vitro by RNAP containing a C-terminal truncation of α , we concluded that TrpI-mediated activation required contact with the region of α defined by the truncation⁴⁴. This would be consistent with the role of TrpI outlined above (step d). However, TrpI is unusual in this respect because other activators whose binding sites are centered at the corresponding position of their target promoters (at -41 to -42 with respect to the transcription startsite) are not affected by the α truncation^{44,53}. Conceivably, rather than abolishing a direct contact between α and TrpI, the truncation abolishes a direct interaction of the α subunit (through its CTD) with far upstream sequences of the promoter and this interaction is required for activation³⁷. This possibility leads to a second model for TrpI-mediated activation.

In Model 2, the steps leading to the establishment of *complex 2* are the same as in Model 1. However, it is imagined that TrpI-induced DNA bending (see below) enables the α -CTD to make contact with an otherwise poorly located upstream sequence. The role of TrpI in this regard would resemble that of IHF in stimulating transcription from the λ *P_{LI}* promoter⁴⁰. In the model, the CTD is thought to be required for activation, not necessarily because of a protein-protein contact between the activator and the CTD, but rather because transcription initiation requires the CTD's interaction with an upstream sequence and this interaction is facilitated by DNA bending. However, the model does not preclude the possibility that direct contact between the CTD and TrpI is also required for activation. In this case, if the CTD and TrpI interact, the interacting TrpI tetramer could be the one bound to site I.

2.2.2. DNA Bending and the Role of Site II in Activation

A 2-bp substitution in site II (called $-10C/11C$) caused a defect in activation of transcription from *trpP_B*, but had very little effect on TrpI binding to site II^{39, 98}. In terms of Model 1, the wild-type nucleotide sequence of site II, which bears little resemblance to site I, might induce in the bound protein a conformation that is essential for subsequent interaction with RNAP to stimulate transcription at *trpP_B* (step d in the scheme outlined above). Such a possibility was suggested originally by Martin *et al.*⁷² based on a mutational analysis of transcriptional activation by AraC and has been supported by several studies of other transcriptional activators (e.g., *Salmonella typhimurium* MetR¹⁴ and the yeast activator PRTF¹¹²).

The phenotype of $-10C/11C$ could be explained (in both Models 1 and 2) by an alternative hypothesis—that TrpI-induced DNA bending, which is required for activation, is somehow affected by the $-10C/11C$ mutation. Direct assays of TrpI-mediated DNA bending⁹⁸ revealed that: (a) TrpI bound to site I (*complex 1*) bends DNA by about 65° (with the center of bending in

the middle of site I); (b) the calculated angle of bending is the same whether *complex 1* is formed on a DNA fragment containing only site I or on a fragment containing both sites; (c) TrpI bound to both sites I and II (*complex 2*) bends DNA by about 85–90° (with the center of bending *between* sites I and II); (d) in no case is the bending angle affected by InGP; (e) the –10C/11C mutation reduces the calculated bending angle of *complex 2* only by about 5°. Thus, the effect of –10C/11C on bending is small enough to suggest (but not prove) that its phenotype does not result from an effect on bending or some other alteration of DNA structure.

2.2.3. *TrpI Binding in the Absence of Site II*

A second mutation (*subI*), an 8-bp substitution, prevents activation of *trpP_B* and abolishes the site II footprint normally made by TrpI³⁹. However, in the presence of InGP, TrpI can form a complex with *subI* DNA whose mobility is similar to that of the corresponding complex (*complex 2*) formed on binding of TrpI to site II on wild-type DNA. This phenotype suggests that a strong cooperative interaction between TrpI tetramers bound to sites I and II can occur even though the second TrpI tetramer is unable to establish intimate contacts with site II DNA (step c in the scheme outlined above). DNA bending studies alluded to above revealed that a different substitution for site II also permits InGP-dependent formation of a complex that migrates in gels with the same mobility as *complex 2*. However, this complex differs from authentic *complex 2*; its center of bending is in the middle of site I and its bending angle is only 75° (ref. [98]).

2.2.4. *Role of Sites I and II*

Although there are numerous cases in which binding of a regulatory protein to a strong binding site facilitates binding to an adjacent, weaker site, in most cases, the protein can bind to the weaker site alone^{59, 114}. Remarkably, TrpI is completely unable to bind to site II in the absence of site I. Nevertheless, hydroxyl radical footprinting identified site II nucleotides that were contacted by TrpI in the presence of InGP¹⁹. The phenotype of the –10C/11C mutation, which alters nucleotides protected by TrpI from hydroxyl radical attack, suggests that these nucleotides are not strong determinants of binding specificity.

A comparison of the intergenic *trpI*–*trpBA* control regions (Figure 3) in seven species in which *trpI* has been identified (see Section 5.1), reveals a putative site I recognition sequence, TGTGAG-N₅-CTGACA (Figure 3), which is highly conserved. Although this sequence is only partially present or not present at all in site II, the region of sequence conservation includes site II⁵. In the four species of fluorescent *Pseudomonads* listed in Figure 3, 18 of 23 bp in site I (including 11 of 12 bp in the putative recognition sequence) are identical in all three species; in site II, 16 of 23 bp are identical. Inclusion of all

ATCTGTGAGTTTTCTGACAGGT	TGCGGCGATCTTATCGGTTTTCA	(P.s.)
ACCTGTGAGTTTTCTGACAAGT	TTGCGCAATCTTATCGGTTTTCA	(P.p.)
ATTCGTGAGTTTTCTGACAGGT	TGAAGCAATCTTATCGGTTTCTT	(P.a.)
ATCTGTGAGTTTTCTGACAGGT	TGTGGCGATCTTATCGGTTTTCA	(P.f.)
ATCGGTGAGTTTTCTGACATAT	TCGGGTGATCTTATCGCTTTTCG	(A.v.)
ATATGTGAGTACAGGTCACAGGT	TGCGGCGATCTTATCGATTTATC	(X.c.)
ATATGTGATTCCAGGTCACAGGT	TGCAGCAATCTTATCGATTTATC	(X.a.)
AtctGTGAGTTTTcCTGACAgGT	TgnnGCnATCTTATCGGTTTTtca	(Fluor.)
AtctGTGAgTtttccTgACAggT	TgngGcnATCTTATCGgTTTTnn	(cons.)

Figure 3. Nucleotide sequences of sites I and II in seven species. Indicated sequences are from top to bottom: *P. syringae*⁵; *P. putida* PpG1 (L. Eberly and Crawford, I. P., unpublished data); *P. aeruginosa* PAC174²¹; *P. fluorescens* PfO-1 (NCBI Acc. No. NZ_AAAUO2000028); *A. vinelandii*¹⁰; *X. campestris*³²; *X. axonopodis*³². The putative TrpI recognition sequence in site I is underlined and italicized as is a possible half sequence in site II. The consensus sequences for the fluorescent species (Fluor.) and for all seven species (cons.) are indicated. Capital letters denote nucleotides present in all seven sequences.

seven species from Figure 3 in the tabulation only slightly reduces the degree of sequence identity: in site I, 11 of 23 bp are conserved; in the putative recognition sequence, 8 of 13 bp are conserved; and in site II, 14 of 23 bp are conserved. These data suggest that site II is constrained in evolution roughly to the same extent as site I. This may be because it specifies the conformation of bound TrpI that is required for activation of *trpP_B*. Alternatively, the sequence of site II may be required to specify the geometry of TrpI-mediated DNA bending, and thus may be a strong determinant of the ability of TrpI to activate *trpP_B*.

2.2.5. InGP Dependence

There is less than a two-fold effect of InGP on the TrpI concentration required for 50% repression of transcription initiation at *trpP_I* in vitro³⁸. Chang and Crawford^{19, 20} argued for a much stronger effect of InGP on TrpI binding to site I (in the absence of RNAP), but their data are difficult to interpret because 100% occupancy of site I was not observed in the absence of InGP.

TrpI binding to site II is strongly stimulated by InGP^{19, 20, 39} but without detailed kinetic data, it is difficult to determine whether InGP affects the degree of cooperativity, the intrinsic affinity of TrpI for site II, or both. However, neither *subI* nor complete substitution of plasmid DNA for site II^{19, 98} prevents InGP-dependent formation of a complex resembling *complex 2*.

When a construct was made containing two tandem copies of site I (either as an inverted or direct repeat), an unexpected result was obtained⁸⁶.

InGP was still required for the formation of *complex 2* and for cooperative binding of TrpI to the two sites. A possible explanation for this result was provided by data that suggested that TrpI binding to two strong sites was actually less efficient than expected because of interference between binding of TrpI tetramers bound to adjacent sites. In this view, InGP could promote formation of *complex 2* by eliminating interference as well as by promoting a cooperative interaction between TrpI tetramers. Thus, InGP stimulates cooperativity whether or not TrpI can establish tight contacts with site II. In contrast, the LysR family member IlvY binds cooperatively to two sites, but cooperativity is unaffected by the inducer, acetohydroxybutyrate¹¹⁴.

Model 2 is attractive because it offers a reasonable explanation of the role of site II and InGP in activation. Two sites may be necessary to achieve the degree of bending required for the CTD to interact with an upstream sequence. Thus, dependence on InGP for binding to site II would allow activation to be regulated in response to changing levels of tryptophan in the cell. When site I was substituted for site II, the calculated bending angle was about 110–115°, nearly twice the bending angle obtained with site I alone, and significantly greater than the bending angle obtained with wild-type sites I and II⁸⁶.

3. REGULATION OF *trpE* AND *trpGDC*

No *trpR* homolog has been identified in fluorescent *Pseudomonads*^{10, 81, 92, 110} or Xanthomonads³², but negative regulation of *trpE* and *trpGDC* by tryptophan has been demonstrated in several species of *Pseudomonas* (Table 2). The isolation of mutants that were resistant to analogs of indole or tryptophan and constitutively expressed *P. putida* and *P. aeruginosa trpE*, *G*, *D*, and *C* suggested that these genes were regulated by a TrpR-like protein^{17, 74}. However, similar constitutive *P. putida* mutants appear to affect attenuation rather than repression⁸⁷ and it is likely that this is the sole mechanism of regulating the *trpE* and *trpGDC* operons in these two species.

Five *P. putida* Tn5-induced mutants, selected for 5-methyltryptophan-resistance and screened for increased expression of *trp* genes⁸⁷, contained insertions in the *P. putida miaA* gene²⁶. The *miaA* gene product participates in modification of adenosines adjacent to the anticodons of several tRNA species, including tRNA^{Trp} (see ref. [33]). In *E. coli*, absence of the modification prevents attenuation¹²¹.

Assays of enzyme levels in prototrophic *P. putida* (Table 2) indicated that, in the presence of excess tryptophan, *miaA* mutations increase expression of *trpE* between 10- and 30-fold and of *trpGDC* between 4- and 8-fold. Thus, inactivating *miaA* indirectly stimulates both *trpE* and *trpGDC* transcription, presumably by inhibiting attenuation. One reason to believe that *trpE* and

trpGDC transcription is regulated only by attenuation is that anthranilate synthase (AS I), phosphoribosyl transferase (PRT), and InGP synthetase (IGPS) levels are roughly the same in a *trpBmiaA::Tn5* double mutant in the presence of excess tryptophan as they are in a *trpB⁻ miaA⁺* strain grown in limiting tryptophan. Furthermore, levels of these enzymes are decreased by at most 40% by added tryptophan in a *trp⁺ miaA::Tn5* mutant.

For *P. putida trpE*, transcription initiates 171 nucleotides upstream from the *trpE* translation initiation codon both in vitro and in vivo⁸⁷. Similar sequences (Figure 4) occur at corresponding positions upstream from *P. putida* and *P. aeruginosa trpE* and *trpGDC*^{34, 35}, and *P. syringae trpE*³¹ and *trpG*¹⁰. These transcripts contain potential leader sequences that encode a very highly conserved polypeptide M(S/R/K)(V/L)IKA(L/H/F)ARWRWRA, which includes two trp residues and is similar to leader peptide sequences identified in other species^{87, 118}. Similar leader peptides are present upstream from *A. vinelandii trpE* (MKVIKALARWRWRA) and *trpG* (MRVVKAHARWRWRA), but they could not be found in *X. axonopodis* or *X. campestris*.

The putative leader transcripts contain several potential attenuator RNA structures^{66, 67}, but the stem-loops are much less stable than those identified in known attenuators. Further complications are the absence, in many cases, of obvious Shine–Dalgarno sequences for initiation of leader peptide synthesis,

trpE

	-35	-10	↗	met	stop	term.?	met (<i>trpE</i>)
<i>P. p.</i>	TTGCGA...N ₁₇ ...	TAACGT...N ₆ ...	A...N ₅₅ ...	ATG...N ₃₉ ...	TGA...N ₄₃ ...	TTATT...N ₂₂ ...	AUG
<i>P. a.</i>	TTGCGC...N ₁₇ ...	TAGTGT...N ₆ ...	A...N ₅₈ ...	ATG...N ₃₉ ...	TGA...N ₄₈ ...	TTCTCT...N ₁₇ ...	AUG
<i>P. s.</i>	TTGCCT...N ₁₇ ...	FACTGT...N ₆ ...	A...N ₅₅ ...	ATG...N ₃₉ ...	TGA...N ₄₁ ...	TTTTT...N ₂₆ ...	AUG

M K V I K A L A R W R W R A

trpGDC

	-35	-10	↗	met	stop	term.?	met (<i>trpG</i>)
<i>P. p.</i>	TTGCGC...N ₁₇ ...	FACTGT...N ₆ ...	A...N ₄₅ ...	ATG...N ₃₉ ...	TGA...N ₄₈ ...	TTTT...N ₈₄ ...	AUG
<i>P. a.</i>	TTGCAC...N ₁₈ ...	TATGTT...N ₇ ...	A...N ₄₅ ...	ACC...N ₃₉ ...	TGA...N ₄₆ ...	TTTT...N ₁₂₀ ...	AUG
<i>P. s.</i>	TTGCAC...N ₁₇ ...	TAATGT...N ₆ ...	A...N ₄₅ ...	ATG...N ₃₉ ...	TGA...N ₄₆ ...	TTTT...N ₁₁₀ ...	AUG

M R V I K A H A R W R W R A
T S L I K A F A R W R W R A

Figure 4. Nucleotide sequences in the putative attenuator regions of *trpE* and *trpGDC* in *Pseudomonas* spp. Nucleotide sequences upstream from the *P. putida* (*P. p.*), *P. aeruginosa* (*P. a.*), and *P. syringae* (*P. s.*) *trpE* and *trpGDC* promoters are shown (see text for references). Amino acid sequences (shown below the nucleotide sequences) are identical for the putative leader peptides preceding *trpE* in all three species. For *trpGDC*, the leader peptides are identical in *P. putida* and *P. syringae*, but the leader peptide (lowest amino acid sequence in figure) is unusual in *P. aeruginosa* (see text). (N_n denotes *n* nucleotides whose sequence is not specified in the figure.)

and in one case (*P. aeruginosa trpG*), the first codon in the putative leader sequence specifies threonine rather than methionine. In all cases, the putative leader peptide forms the C-terminus of a longer open reading frame. An unusual feature of the upstream region of *trpE* in these three species is that the putative leader peptide is the carboxy terminus of a reading frame homologous to phosphoglycolase phosphatase (Gph) of *E. coli*^{34, 35} (GenBank, AB030825), although in the *E. coli* genome *gph* is not upstream of *trpE*⁸.

While it seems safe to conclude that attenuation is the primary, if not the sole, means of regulating these two operons, the details of the mechanism have not been elucidated. Direct proof that the putative attenuator is functional and regulated by tryptophan would require additional evidence, including, for example, the identification of the terminated leader transcript and demonstration that it is extended under starvation conditions in *trp* auxotrophs.

Note that in *Rhizobium meliloti*, only the *trp(E•G)* fusion gene is regulated (by attenuation)⁶, and even in auxotrophic *Rhizobium leguminosarum*, expression of the other *trp* genes is not significantly affected by exogenous tryptophan⁴⁹.

4. HOW THE PATHWAY WORKS

4.1. Role of *trpI*

The *trpI*-mediated regulatory circuit is logically the same as that in species in which *trpB* and *trpA* are repressed by TrpR. InGP is produced (and *trpP_B* is activated) only when *trp* genes whose products function earlier in the pathway are expressed. In wild-type cells (Table 2), endogenous tryptophan levels appear to be sufficient to substantially reduce expression of both *trpE* and *trpGDC*, presumably by attenuation. Thus, addition of exogenous tryptophan results in only a 10–25% decrease in expression of these genes. This is to be contrasted with the effect of limiting tryptophan on *trpBA* transcription, where even in prototrophs growth in limiting tryptophan reduces levels of tryptophan synthase by a factor of 5–10 (Table 2). This is most likely due to feedback regulation of anthranilate synthase, which inhibits the conversion of chorismate to anthranilate, resulting in failure to synthesize InGP. Thus, in wild-type cells, the most dramatic effect of tryptophan on *trp* gene expression is mediated by a combination of feedback inhibition and the need for InGP and TrpI to activate *trpBA*.

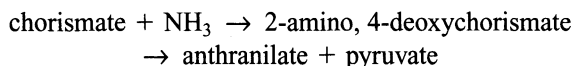
In experiments with *trp* auxotrophs, endogenous and exogenous tryptophan are depleted, thus permitting substantially increased expression of most *trp* genes through relief of attenuation. Under these conditions, increased expression of *trpE* and *trpGDC* is substantial and—when InGP synthesis

is not blocked—the increase in expression of *trpBA* is even more dramatic (Table 2).

4.2. Feedback Inhibition

As has been alluded to previously, tryptophan regulates both the transcription of *trp* genes and the activity of anthranilate synthase, the first enzyme devoted exclusively to tryptophan biosynthesis. Feedback inhibition of anthranilate synthase is the most immediate and probably most significant cellular response to increased tryptophan levels in prototrophic fluorescent *Pseudomonads* since the effect of tryptophan on synthesis of the first four enzymes in the biosynthetic pathway is minimal (Table 2).

Anthranilate synthase is an oligomer of the form $\alpha\beta$ in fluorescent *Pseudomonads* (*Pseudomonas putida*, *Pseudomonas aeruginosa*, and *Pseudomonas stutzeri*) and $\alpha_2\beta_2$ in several other species, including *C. acidovorans*, *P. testosteroni*, and *B. cepacia*¹⁰¹ and some enteric species^{80, 108}. The larger (α) subunit (component I) is the product of *trpE*, while the smaller (β) subunit (component II) is the product of *trpG*. The conversion of chorismate to anthranilate can be carried out in the presence of NH_3 by the TrpE subunit alone, but the addition of component II (TrpG) allows the enzyme to obtain NH_3 through the deamination of glutamine to glutamate. Although component II contains the binding site for glutamine, glutamine binds only to the intact oligomer¹⁰⁰. Whether it is catalyzed by component I alone or by the $\alpha\beta$ oligomer, the synthesis of anthranilate (Figure 1) is actually a two-step reaction^{79, 82}:



The first step, catalyzed by the aminodeoxyisochorismate (ADIC) synthase activity of the oligomeric protein, involves addition of an amino group and removal of the hydroxyl group from the chorismate ring structure. In the second step, the ADIC lyase activity removes the enol-pyruvate side chain as pyruvate and “aromatizes” the ring structure to produce anthranilate (= o-aminobenzoate). It has been known for some time that the site of feedback regulation is in component I¹⁰⁰. X-ray structural studies of the tetrameric enzyme from *Serratia mercescans*¹⁰⁸ and *S. typhimurium*⁸⁰ have revealed the sites of binding of chorismate, glutamate, and tryptophan. Even though the sites for tryptophan and chorismate (on component I) do not overlap, inhibition by tryptophan is competitive^{100, 124} because the enzyme can be converted from an intermediate form to either of two mutually exclusive ligand-bound forms¹⁰⁸. The enzymatically active form is induced by binding of chorismate, while the inactive form is induced when the intermediate binds tryptophan.

Thus, each ligand competitively inhibits binding of the other by altering the structure of the enzyme. Inhibition of binding of glutamine to component II by tryptophan, shown previously to be noncompetitive¹⁰⁰, is mediated by an overall change in conformation of the tetramer when tryptophan binds to component I.

4.3. *trpF* Regulation

In fluorescent *Pseudomonads*, phosphoribosyl anthranilate isomerase (PRAI), the product of *trpF*, is expressed at fairly low levels both in the presence and absence of tryptophan (Table 2). Calhoun *et al.*¹⁷ and Crawford²⁷ pointed out that the level of PRAI normally seen in minimal medium seemed sufficient to rapidly process available phosphoribosyl anthranilate. However, in fully derepressed cells (e.g., in mutants resistant to tryptophan analogs), phosphoribosyl anthranilate accumulates, spontaneously decomposes, and is excreted as anthranilate¹⁷. Thus, it is conceivable that even in derepressed wild-type cells PRAI levels regulate the synthesis of InGP and thereby indirectly limit expression of *trpBA*.

4.4. DAHP Synthase

Figure 1 illustrates the sequence of reactions exclusively used in the synthesis of tryptophan from CHA. However, the shikimic acid pathway (not shown in Figure 1) is required for the synthesis of CHA. The key (first) step in this pathway—the synthesis of deoxy-D-*arabino*-heptulosonic acid-7-phosphate (DAHP) from erythrose-4-phosphate and phosphoenolpyruvate—is catalyzed by one or more isoforms of DAHP synthase, whose synthesis and/or activity are regulated in most bacterial species by one or more of the end-products of aromatic amino acid biosynthesis (tyrosine, phenylalanine, and tryptophan). In *E. coli* (and all enterics), three paralogous isoforms^{1, 2, 56}, encoded by *aroF*, *aroG*, and *aroH*, are subject to feedback inhibition by tyrosine, phenylalanine, and tryptophan, respectively^{58, 99}. In addition, *aroF* and *aroG* are repressed by TyrR in the presence of tyrosine or phenylalanine, respectively^{9, 18, 54}, while *aroH* is repressed by the *trpR* repressor in the presence of tryptophan^{65, 126}.

In fluorescent *Pseudomonads*^{15, 16, 85, 116}, two main isoforms of DAHP synthase are found in exponential cultures. They correspond functionally and evolutionarily to AroF and AroH of *E. coli*. The major (AroF-like) isoform (90% of total activity) is inhibited by tyrosine while the minor (AroH-like) isoform (10% of total activity) is inhibited by tryptophan.

It is not clear whether or not *aroF* and *aroH* in fluorescent *Pseudomonads* are regulated transcriptionally. Jensen and coworkers found that in all enterics studied, all three DAHP isoforms were subject to transcriptional control², but they found no evidence for transcriptional control in group I

(fluorescent) *Pseudomonads*^{2, 116}. However, repression of *aroF* by tyrosine and phenylalanine in *P. aeruginosa*, *P. putida*, and *P. fluorescens* was reported by Olekhovich *et al.*⁸⁹ and Maksimova *et al.*⁷⁰. Repression was not observed for the related species *P. stutzeri* and *P. mendocina* and contradictory results were obtained for two different strains of *P. fluorescens*⁸⁹. In order to observe regulation of DAHP synthases in the absence of endogenous tyrosine, tryptophan, and phenylalanine, *aro* mutants, blocked at unknown step(s) in the shikimic acid pathway were independently isolated for each strain tested⁸⁹. Conceivably, in some cases, the putative mutation was leaky, permitting repression of *aroF* even in the absence of added amino acid(s). (This explanation requires that a corepressor, tyr and/or phe, be synthesized in sufficient quantities to cause repression, but not to permit growth on minimal medium.)

One way to resolve this question would be to find a true homolog of TyrR in fluorescent *Pseudomonads*. A BLAST search of the NCBI database yields numerous potential homologs in the genomes of *P. syringae*, *P. putida*, and *P. aeruginosa*, but the extent of sequence agreement with *E. coli* TyrR is too low to be able to predict the normal function of each of the homologs in the absence of direct genetic or biochemical evidence. The highest ranking TyrR homolog identified in a BLAST search is *P. aeruginosa* PhhR, which is a positive regulator of an operon encoding phenylalanine hydroxylase; this enzyme, which among the γ -proteobacteria is unique to group I *Pseudomonads*, produces tyrosine from phenylalanine and permits cell growth on phenylalanine or tyrosine as sole carbon source. *P. aeruginosa* PhhR, which shares 47% amino acid sequence identity with *E. coli* TyrR, can repress the *aroF* operon in *E. coli*, but mutating *phhR* had no effect on DAHP synthase expression in *P. aeruginosa*¹⁰⁷.

Even in studies in which repression of *aroF* by tyrosine or phenylalanine was demonstrated, there was no evidence for repression of the paralogous gene, *aroH*, in fluorescent *Pseudomonads*. This would be difficult to demonstrate without testing each gene individually, since, as mentioned previously, the trp-sensitive isoform constitutes only about 10% of the total DAHP synthase in the cell. For the same reason, the need for repression of *aroH* should be negligible.

5. EVOLUTION OF KEY PATHWAY COMPONENTS

5.1. TrpI

A survey of the NCBI genome database reveals a homolog to *trpI* in six different strains of *P. putida*, *P. aeruginosa*, and *P. syringae* (all those whose sequences have been determined)^{5, 10, 19, 21, 81, 110}, and in four other

Table 3. Degree of amino acid identity among known TrpI sequences^{a,b}.

	<i>P. putida</i>	<i>P. fluor.</i>	<i>A. vinel.</i>	<i>P. aerug.</i>	<i>X. camp.</i>	<i>X. axon.</i>
<i>P. syringae</i> ^c	79.9	81.6	68.1	69.5	58.7	54.5
<i>P. putida</i>		76.4	72.8	70.0	60.4	57.1
<i>P. fluorescens</i> ^d			67.9	67.5	57.7	54.5
<i>A. vinelandii</i> ^e				73.2	60.1	56.0
<i>P. aeruginosa</i>					57.0	55.5
<i>X. campestris</i>						75.3

^aPercentage of identical amino acids (number of identities divided by length of longer of the two sequences).

^bSequences used: *P. syringae* strain PS224⁵; *P. putida* PpG1. (Eberly and Crawford, unpublished data; see ref. [5]); *P. fluorescens* PfO-1 (NCBI Accession no. ZP_00086671.1); *A. vinelandii* (NCBI Accession no. NZ_AAAU02000028); *P. aeruginosa* PAC174²¹; *X. campestris* pv. *campestris* str. ATCC 33913³²; *X. axonopodis* pv. *citri* str. 306³².

^cThe sequences of two *P. syringae* strains (PS224 and pv. tomato strain DC3000, ref. [10]) differ from each other to a greater extent than do sequences of separate strains of *P. aeruginosa* and *P. putida*. For TrpI from the two *P. syringae* strains, the degree of sequence identity is 80.4%.

^dAlignment of *P. fluorescens* TrpI required deletion of an A:T bp and one of three G:C bp, which are located 77 bp and 16, 17, or 18 bp preceding the inferred translation start codon (GUG) in the NCBI sequence (see text).

^eThe NCBI sequence includes 20 additional amino acids in the correct reading frame at the N-terminus of the inferred amino acid sequence; these amino acids were excluded from the calculation of sequence identity.

species: *P. fluorescens* (NCBI accession no. AABAO1000149) *A. vinelandii* (NCBI accession no. NZ_AAAUO2000028), *X. axonopodis*, and *X. campestris*³². The criteria for concluding that these homologs are authentic *trpI* are the following: (a) They are adjacent to, and transcribed divergently from, homologs to *trpB* and *trpA*; (b) the inferred amino acid sequence of each putative TrpI shares at least 55% sequence identity with TrpI of the three species of fluorescent Pseudomonads in which it was originally discovered (Table 3). (c) The intergenic control region containing sites I and II shares significant nucleotide sequence identity with the corresponding sites in the three original species (Figure 3). Non-TrpI LysR family members were identified by a BLAST search as homologs of TrpI, but these share no more than 38% sequence identity with putative TrpI proteins and do not share the prototypical gene arrangement (*trpI-trpBA*). Whether the newly-identified *trpI* genes are functional remains to be determined. Indeed, the *P. fluorescens* PfO-1 sequence in the database is a truncated version that is missing 87 amino acids from its N-terminus. However, the entire TrpI sequence can be deduced if 2 bp (separated by 60 bp within the N-terminal domain) are deleted. The two "extra" bp could reflect sequencing error or the possible conversion of *trpI* into a pseudogene through genetic drift.

The phylogenetic relationships among these species has been extensively investigated^{4, 56}. Abbreviated versions of two alternative phylogenetic

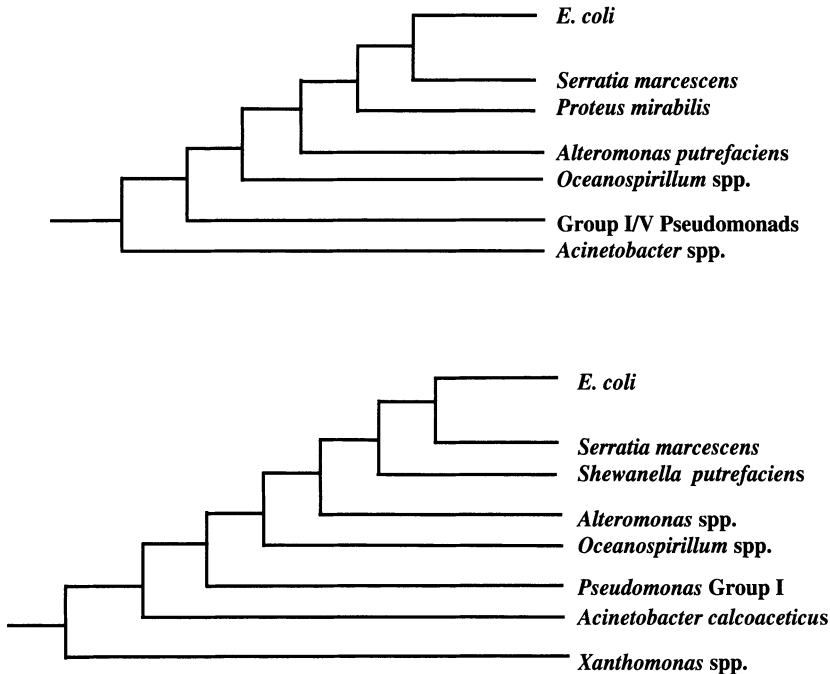


Figure 5. Phylogenetic relationships among γ -proteobacteria. Abbreviated phylogenies, based on 16S rDNA sequences, are taken from ref. [56] (upper) and ref. [4] (lower). In the upper phylogeny, the branches corresponding to fluorescent Pseudomonads (group I) and *Xanthomonas* spp. (group V) are merged.

trees that have been presented (Figure 5) reveal an interesting difference. Both trees were deduced from 16S rDNA sequences, but the lower tree reflects a much larger data set⁴. In this tree, the earliest branch point in the γ subdivision of the proteobacteria separates the *Xanthomonas* group from all other branches, including an early branch that includes *A. calcoaceticus*. In the upper tree⁵⁶, *A. calcoaceticus* diverges at the first branchpoint and *Xanthomonads* are placed in the same branch as the fluorescent Pseudomonads (Figure 5). Precisely where the *Xanthomonads* and fluorescent Pseudomonads diverge is not so important for this discussion as the question of their position relative to that of *A. calcoaceticus*.

The presence of a *trpI* homolog in genomes of two *Xanthomonas* species³² but its absence, at least according to physiological data (Table 2), in *A. calcoaceticus* makes some version of the upper tree in Figure 5 (see ref. [56]) appear to be more likely, since the easiest explanation is that *trpI* arose after the divergence of the "group I/V pseudomonads" from a common ancestor, but

before the separation of group I (fluorescent *Pseudomonads*) from group V (*Xanthomonas* group). The existence of *trpI* in *A. vinelandii* is consistent with either phylogenetic tree, since the rRNA sequence homology between *A. vinelandii* and *P. aeruginosa* is approximately 96% (see ref. [4]), which would place *A. vinelandii* in group I according to the classification of Palleroni *et al.*⁹⁰.

The data in Table 1 suggest that at one point *trpF*, *trpB*, and *trpA* were tightly linked, and that *trpI* may have arisen following insertion of an ancestral *lysR*-like gene between genes *B* and *F*. Subsequently, *trpIBA* would have been kept together because both the *trpBA* and *trpI* promoters overlap the TrpI binding sites (Figure 2), but since *trpF* is unregulated its position could be completely independent of the positions of the other *trp* genes.

Palleroni's original scheme⁹⁰ subdivided the *Pseudomonads* into five groups. In addition to those already mentioned, groups II and III are in the β subdivision and Group IV is in the α subdivision of the proteobacteria. Many of the original species names have been changed from *Pseudomonas* spp. to *Burkholderia* spp. or *Ralstonia* spp. (Group II), *Comamonas* spp. (Group III), or *Brevundimonas* spp. (Group IV). Very little work has been done on gene regulation in these taxa, but data obtained for the β subdivision species, *C. acidovorans*, indicate that it does not have *trpI* (Table 2).

Two additional species, *P. mendocina* (group I) and *Pseudomonas marginata* (group II), were examined through the isolation and characterization of *trp* auxotrophs⁶³. In neither species was there evidence for regulation of *trpB* by tryptophan. Absence of *trpI* in a member of group II (*P. marginata*) is not surprising, but since *P. mendocina* is closely related to *P. aeruginosa*⁴, the absence of *trpI* in this species is unexpected. However, the evidence on which this conclusion is based comes primarily from the phenotype of a single *trpA* mutant. Clearly, additional genomic data will be necessary to verify this result and ultimately to help determine the evolutionary origin of *trpI*.

5.2. Anthranilate Synthase

Anthranilate synthases are part of an intriguing web of inter-related enzymatic activities that are especially relevant to considerations of the way related enzymes can evolve through gene duplication, fusion, and divergence⁸². The synthesis of p-aminobenzoic acid (PABA) from chorismate is analogous biochemically to the synthesis of anthranilate (o-aminobenzoic acid)^{82, 83}. In fact, some of the gene products involved are clearly homologous^{30, 82}. Although both the synthase and lyase activities of anthranilate synthase are encoded in a single protein, the two-step synthesis of PABA requires two separate enzymes. The first enzyme (the synthase) consists of two subunits, PabA and PabB, which are homologs of TrpG and TrpE, respectively^{41, 61}, but are unable by themselves to perform the lyase function characteristic of

anthranilate synthase. Lyase activity requires the product of a third gene, *pabC*⁴³. In spite of the need for *pabC*, the two gene pairs, *trpG/trpE* and *pabA/pabB* are in fact homologous. Both pairs of genes are found in *E. coli* and other enterics, but in at least three species (*Bacillus subtilis*, *A. calcoaceticus*, and *C. acidovorans*), the glutamine amidotransferase function (*trpG/pabA*) of both synthases is the product of the same gene^{13, 60, 104}.

In *P. aeruginosa*, this situation is even more complicated because of the existence of a second anthranilate synthase, encoded by genes *phnA* and *phnB*. This anthranilate synthase was originally thought to be part of the pathway leading to the synthesis of the fluorescent compound pyocyanin (a derivative of phenazine)³⁰. Starting with a *trpE* knockout mutant, it was possible to select revertants able to grow in the absence of tryptophan. These revertants turned out to be over-expressors of *phnA* and *phnB*. Their survival indicates that under conditions of over-expression, but not in wild-type cells, *phnA* and *phnB* produce substantial quantities of anthranilate that can serve as precursor to tryptophan.

However, the actual role of *phnA* and *phnB* in *P. aeruginosa* has been called into question by the identification of a cluster of seven genes (*phzA-G*) responsible for phenazine biosynthesis in *Pseudomonas aureofaciens*⁹⁷, *Pseudomonas fluorescens*⁷⁵, and *Pseudomonas chlororaphis*²³ and of two nearly-identical clusters in *P. aeruginosa*⁷⁶. Each cluster contains a gene, *phzE*, that resembles a *trpE*•*trpG* fusion and is responsible for the conversion of chorismate to 3-hydroxyanthranilate. Thus, anthranilic acid is apparently not an intermediate in the formation of phenazine. Furthermore, Mavrodi *et al.*⁷⁶ presented evidence suggesting that *phnAB* is not directly involved in phenazine synthesis when the *phzA-G* pathway is active. Still, there is agreement that *phnAB* in some mysterious way influences pyocyanin synthesis⁷⁶.

Regardless of the actual biological role of *phnAB*, the degree of sequence similarity among various homologs²⁸, particularly of *trpG*, *phnB*, and *pabA* led Crawford and Milkman³⁰ to propose an unexpected pattern of evolution of the corresponding genes: (a) It was proposed that *trpG_o*-*trpE_o*, which existed before the divergence of the γ -proteobacteria, participated in the synthesis of both anthranilate and PABA. (b) Duplication of these genes early in the γ -proteobacteria lineage produced two gene pairs: *trpG₁*-*trpE₁* and *trpG₂*-*trpE₂*. (c) In the enterics, *trpG₁*-*trpE₁* became modern-day *trpG* and *trpE*, while *trpG₂*-*trpE₂* became modern-day *pabA* and *pabB*. In *P. aeruginosa*, *trpG₂*-*trpE₂* became modern-day *trpG* and *trpE*, while *trpG₁*-*trpE₁* became modern-day *phnB* and *phnA*. Thus, *trpG* of *P. aeruginosa* is more closely related to *pabA* than to *trpG* of the enterics and *phnB* of *P. aeruginosa* is more closely related to *trpG* than to *pabA* of the enterics^{28, 82}.

Recently, the structure of PabB of *E. coli* was shown to contain a tightly-bound tryptophan molecule in precisely the site at which tryptophan mediates

feedback regulation of the TrpE subunit of anthranilate synthase(s)⁹⁴. Since tryptophan does not regulate the activity of PABA synthase, its presence in crystals of PabB may reflect evolutionary modification of its role as a feedback inhibitor to an essential role in maintaining the structure of the enzyme.

A particularly puzzling question is what provides *pabA*, *B*, and *C* function for *P. aeruginosa*. A *phnA-trpE* double mutant was found to grow normally on minimal medium supplemented with tryptophan³⁵, strongly suggesting that neither gene product is essential for PABA synthesis in *P. aeruginosa*. Yet, the genomes of *P. aeruginosa*, *P. putida*, and *P. syringae* contain no obvious *pabB*, *pabA*, or *pabC* homologs. Given the apparent homology switch suggested by Crawford and Milkman³⁰, it can not be certain from amino acid sequence homology alone, which function a particular homolog might actually serve.

5.3. DAHP Synthase

Jensen and coworkers suggested an intriguing scenario for the evolution of DAHP isoforms in the γ -proteobacteria^{56, 57}. It was proposed that an ancestor preceding the divergence of the γ -proteobacteria possessed two isoforms of DAHP synthase, called DS-0 and DS-Y. The former was not subject to feedback inhibition, while the latter was inhibited by tyrosine. These forms are present in two of the earliest species in this group, *Oceanospirillum minutulum* and *A. calcoaceticus* (only *A. calcoaceticus* is shown in Figure 5). At some point prior to divergence of the fluorescent Pseudomonads and, according to Jensen's hypothesis, the Xanthomonads, DS-0 acquired responsiveness to tryptophan and CHA and became DS-W. Subsequently, DS-W was duplicated to produce the third isoform, DS-F. These three isoforms are found in all enteric lineages, though there is variable responsiveness of DS-W to CHA and in some cases, the response to CHA is much more pronounced than the response to tryptophan. To complete the scenario, it was hypothesized that, in group V Pseudomonads (*Xanthomonas* spp.), DS-Y had been lost and DS-W had become more sensitive to CHA than to tryptophan¹¹⁵.

However, it is now apparent that the evolution of DAHP synthases is much more complicated. There are, in fact, two major families, called AroA_I and AroA_{II}. The AroA_I family is largely composed of those synthases that are used in aromatic amino acid biosynthesis, including the two isoforms in fluorescent Pseudomonads and the three isoforms in enterics. The AroA_{II} family includes enzymes specified by the *phzC* genes in the phenazine gene clusters of *P. aeruginosa* and other fluorescent Pseudomonads, as well as homologs (of bacterial origin) found in plants and fungi^{42, 111}. Most of the group II enzymes are not feedback inhibited; in bacteria, the *phz* operon is expressed only in stationary phase, and may be subject to quorum-sensing control^{76, 97, 117}.

The interesting fact is that in several species, including *X. campestris*, the AroA_{II} enzyme, the only isoform present, has acquired a binding site for feedback regulation, is no longer restricted in its synthesis to stationary phase, and must be responsible for biosynthesis of aromatic amino acids⁴². At least in the case of *X. campestris*, it thus appears that previously existing AroA_I paralog(s) have been lost and have been functionally replaced by a pre-existing AroA_{II} enzyme. A phylogeny of the DAHP homologs representing both families⁴² reflects potential problems—including gene duplication, gene loss, gene fusion, and perhaps lateral transfer—that complicate attempt to understand bacterial gene evolution.

Gene loss within a paralogous set⁴² is also apparent in the existence of only one DAHP synthase in *H. influenzae*. In this unusual case, there is a possible TrpR binding site upstream from the corresponding gene⁹², but the feedback inhibition site of the inferred gene product resembles that of the phenylalanine-sensitive enzyme, DS-F (AroG).

6. CONCLUSIONS

An extensive survey of tryptophan biosynthetic enzymes and the genes that encode them led to a key conclusion^{28,48}: The enzymes in the biosynthetic pathway are much more highly conserved than the arrangement of the corresponding genes in transcription units or the mechanisms by which expression of the genes is regulated. Largely because of pioneering comparative nucleotide sequence studies and enzymologic data, the organization of *trp* genes and regulation of their expression have provided unparalleled opportunities to explore the evolution of a paradigmatic metabolic pathway^{15, 28, 30, 56}.

Speculation about the importance of TrpI to fluorescent *Pseudomonads* leads to several potentially interesting conclusions. First, it has been suggested¹²⁰ that repression of *trp* genes in *E. coli* and other enterics came late in evolution—that TrpR's original function was regulation of *aroH*, which encodes one of the paralogous enzymes essential for the first step in the synthesis of chorismate, and thus is a key enzyme in tryptophan biosynthesis. Presumably, a selective advantage for the evolution of a *trp* operator would be greatest in cells in which the *trp* genes were transcribed as a single unit. Alternatively, repression in enterics might have evolved initially to control synthesis of only a single gene (e.g., *trpE*). Fusion of additional *trp* genes (especially the closely-linked *trpGDC* operon) to *trpE* would have allowed for coordinate regulation, ultimately leading to the arrangement we see in *E. coli* and its close relatives. Interestingly, *Hemophilus influenzae*, a member of the Pasteurella group^{4,42}, contains two separate *trp* operons, *trpEGDC* and *trpBA*. On the other hand, the genes exist in a single operon in *H. influenzae*'s very close relative, *Pasteurella multocida*^{4,42}.

Failure to evolve repression, by analogy with *E. coli*, would prevent regulation of any part of the pathway at the transcriptional level except under conditions of extreme starvation for tryptophan. Thus, TrpI-mediated regulation of *trpBA* expression may have provided a selective advantage by allowing these genes to respond to tryptophan depletion that is not so severe as that required for relief of attenuation.

Since *trpP_B* and the TrpI-binding sites overlap and InGP has a modest effect on TrpI binding to site I *in vitro*³⁸, TrpI will turn down its own synthesis at the same time that it activates the *trpBA* operon. (The pattern of regulation is similar for several other LysR family members, e.g., NahR¹⁰⁶, IlvY¹¹⁴, and MetR¹¹³.) Apparently, TrpI levels are programmed to be *higher* when tryptophan is present and InGP levels are low; thus, the *trpBA* operon will be poised to respond rapidly to depletion of tryptophan, relief of feedback inhibition, and increased InGP levels. Conversely, in the absence of tryptophan, increased synthesis of InGP would increase TrpI binding to site I, thereby reducing TrpI levels. This would permit the *trpBA* operon to be turned off more rapidly once tryptophan levels were restored (and the InGP concentration decreased).

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REFERENCES

1. Ahmad, S., Rightmire, B., and Jensen, R.A., 1986, Evolution of the regulatory isozymes of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase present in the *Escherichia coli* genealogy. *J. Bacteriol.*, 165:146–154.
2. Ahmad S., Johnson J.L., and Jensen, R.A., 1987, The recent evolutionary origin of the phenylalanine-sensitive isozyme of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase in the enteric lineage of bacteria. *J. Mol. Evol.*, 25:159–167.
3. Ansari, A.Z., Bradner, J.E., and O'Halloran, T.V., 1995, DNA-bend modulation in a repressor-to-activator switching mechanism. *Nature*, 374:371–375.
4. Anzai, Y., Kim, H., Park, J.-Y., Wakabayashi, H., and Oyaizu, H., 2000, Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. *Int. J. Syst. Evol. Microbiol.*, 50:1563–1589.
5. Auerbach, S., Gao, J., and Gussin, G.N., 1993, Nucleotide sequences of the *trpI*, *trpB*, and *trpA* genes of *Pseudomonas syringae*: Positive control unique to fluorescent pseudomonads. *Gene*, 123:25–32.

6. Bae, Y.M. and Crawford, I.P., 1990, The *Rhizobium meliloti trpE(G)* gene is regulated by attenuation, and its product, anthranilate synthase is regulated by feedback inhibition. *J. Bacteriol.*, 172:3318–3327.
7. Blatter, E.E., Ross, W., Gourse, R.L., and Ebright, R.H., 1994, Domain organization of RNA polymerase α subunit: The C-terminal 85 amino acids constitute a domain capable of dimerization and DNA binding. *Cell*, 78:889–896.
8. Blattner, F.R., Plunkett III, G., Bloch, C.A., Perna, N.T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J.D., Rode, C.K., Mayhew, G.F., Gregor, J., Davis, N.W., Kirkpatrick, H.A., Goeden, M.A., Rose, D.J., Mau, B., and Shao, Y., 1997, The complete genome sequence of *Escherichia coli* K-12. *Science*, 277:1453–1474.
9. Brown, K.D. and Somerville, R.L., 1971, Repression of aromatic amino acid biosynthesis in *Escherichia coli* K-12. *J. Bacteriol.*, 108:386–389.
10. Buell, R., Joardar, V., Khouri, H., Fedorova, N., Tran, B., Russell, D., Berry, K., Utterback, T., Van Aken, S., Feldblyum, T., Gwinn, M., Dodson, R., DeBoy, R., Durkin, A., Kolonay, J., Madupu, R., Daugherty, S., Brinkac, L., Beanan, M., Haft, D., Selengut, J., Nelson, W., Davidsen, T., White, O., Fraser, C., and Collmer, A., 2003, GenBank Accession No. AE016853.
11. Busby, S. and Ebright, R., 1994, Promoter structure, promoter recognition, and transcription activation in prokaryotes. *Cell*, 79:743–746.
12. Busby, S. and Ebright, R.H., 1999, Transcription activation by catabolite activator protein (CAP). *J. Mol. Biol.*, 293:199–213.
13. Buvinger, W.E., Stone, L.C., and Heath, H.E., 1981, Biochemical genetics of tryptophan synthesis in *Pseudomonas acidovorans*. *J. Bacteriol.*, 147:62–68.
14. Byerly, K.A., Urbanowski, M.L., and Stauffer, G.V., 1991, The MetR binding site in the *Salmonella typhimurium metH* gene: DNA sequence constraints on activation. *J. Bacteriol.*, 173:3547–3553.
15. Byng, G.S., Johnson, J.L., Whitaker, R.J., Gherna, R.L., and Jensen, R.A., 1983, The evolutionary pattern of aromatic amino acid biosynthesis and the emerging phylogeny of Pseudomonad bacteria. *J. Mol. Evol.*, 19:272–282.
16. Byng, G.S., Berry, A., and Jensen, R.A., 1983, A pair of regulatory isozymes for 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase is conserved within group I Pseudomonads. *J. Bacteriol.*, 156:429–433.
17. Calhoun, D.H., Pierson, D.L., and Jensen, R.A., 1973, The regulation of tryptophan biosynthesis in *Pseudomonas aeruginosa*. *Mol. Gen. Genet.*, 121:117–132.
18. Camakaris, H. and Pittard, J., 1973, Regulation of tyrosine and phenylalanine biosynthesis in *Escherichia coli* K-12: Properties of the *tyrR* gene product. *J. Bacteriol.*, 115:1135–1144.
19. Chang, M. and Crawford, I.P., 1990, The roles of indoleglycerol phosphate and the TrpI protein in the expression of *trpBA* from *Pseudomonas aeruginosa*. *Nucleic Acids Res.*, 18:979–988.
20. Chang, M. and Crawford, I.P., 1991, In vitro determination of the effect of indoleglycerol phosphate on the interaction of purified TrpI protein with its DNA binding sites. *J. Bacteriol.*, 73:1590–1597.
21. Chang, M., Hadero, A., and Crawford, I.P., 1989, Sequence of the *Pseudomonas aeruginosa trpI* activator gene and relatedness of *trpI* to other procaryotic regulatory genes. *J. Bacteriol.*, 171:172–183.
22. Chen, Y., Ebright, Y., and Ebright, R., 1994, Identification of the target of a transcription activator protein by protein–protein photocrosslinking. *Science*, 265:90–92.
23. Chin-A-Woeng, T., 2000, Molecular basis of biocontrol of tomato foot and root rot by *Pseudomonas chlororaphis* strain PCL1391. Ph. D. thesis. University of Leiden, Leiden, The Netherlands.

24. Claverie-Martin, F. and Magasanik, B., 1991, Role of integration host factor in the regulation of the *glnHp2* promoter of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA*, 88:1631–1635.
25. Cohn, W. and Crawford, I.P., 1976, Regulation of enzyme synthesis in the tryptophan pathway of *Acinetobacter calcoaceticus*. *J. Bacteriol.*, 127:367–379.
26. Connolly, D.M. and Winkler, M.E., 1989, Genetic and physiological relationships among the *miaA* gene, 2-methylthio-N⁶-(Δ^2 -isopentenyl)-adenosine tRNA modification, and spontaneous mutagenesis in *Escherichia coli* K-12. *J. Bacteriol.*, 171:3233–3246.
27. Crawford, I.P., 1986, Regulation of tryptophan synthesis in *Pseudomonas*. In J. Sokatch (ed.), *The Bacteria*, vol. 10, pp. 251–263. Academic Press, New York, NY.
28. Crawford, I.P., 1989, Evolution of a biosynthetic pathway: The tryptophan paradigm. *Annu. Rev. Microbiol.*, 43:567–600.
29. Crawford, I.P. and Gunsalus, I.C., 1966, Inducibility of tryptophan synthetase in *Pseudomonas putida*. *Proc. Natl. Acad. Sci. USA*, 56:717–724.
30. Crawford, I.P. and Milkman, R., 1990, Orthologous and paralogous divergence, reticulate evolution, and lateral gene transfer in bacterial *trp* genes. In R.K. Selander, A.G. Clark, and T.S. Whittam (ed.), *Evolution at the Molecular Level*, pp. 77–95. Sinauer Assoc., Sunderland, MA.
31. da Costa e Silva, O. and Kosuge, T., 1991, Molecular characterization and expression analysis of the anthranilate synthase gene of *Pseudomonas syringae* subsp. *savastanoi*. *J. Bacteriol.*, 173:463–471.
32. da Silva, A.C.R., Ferro, J.A., Reinach, F.C., Farah, C.S., Furlan, L.R., Quaggio, R.B., Monteiro-Vitorello, C.B., Van Sluys, M.A., Almeida N.F., Jr, Alves, L.M.C., do Amaral, A.M., Bertolini, M.C., Camargo, L.E.A., Camarotte, G., Cannavan, F., Cardozo, J., Chamberg, F., Ciapina, L.P., Cicarelli, R.M.B., Coutinho, L.L., Cursino-Santos, J.R., El-Dorry, H., Faria, J.B., Ferreira, A.J.S., Ferreira, R.C.C., Ferro, M.I.T., Formighieri, E.F., Franco, M.C., Greggio, C.C., Gruber, A., Katsuyama, A.M., Kishi, L.T., Leite, R.P., Jr, Lemos, E.G.M., Lemos, M.V.F., Locali, E.C., Machado, M.A., Madeira, A.M.B.N., Martinez-Rossi, N.M., Martins, E.C., Meidanis, J., Menck, C.F.M., Miyaki, C.Y., Moon, D.H., Moreira, L.M., Novo, M. T. M., Okura, V.K., Oliveira, M.C., Oliveira, V.R., Pereira H.A., Jr, Rossi, A., Sena, J.A.D., Silva, C., de Souza, R.F., Spinola, L.A.F., Takita, M.A., Tamura, R.E., Teixeira, E.C., Tezza, R.I.D., Trindade dos Santos, M., Truffi, D., Tsai, S.M., White, F.F., Setubal, J.C. and Kitajima, J.P., 2002, Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. *Nature*, 417:459–463.
33. Eisenberg, S.P., Yarus, M., and Soll, L., 1979, The effect of an *Escherichia coli* regulatory mutation on transfer RNA structure. *J. Mol. Biol.*, 135:111–126.
34. Essar, D.W., Eberly, L., and Crawford, I.P., 1990, Evolutionary differences in chromosomal location of four early genes of the tryptophan pathway in fluorescent pseudomonads: DNA sequences and characterization of *Pseudomonas putida trpE* and *trpGDC*. *J. Bacteriol.*, 172:853–866.
35. Essar, D.W., Eberly, L., Han, C.-Y., and Crawford, I.P., 1990, DNA sequences and characterization of four early genes of the tryptophan pathway of *Pseudomonas aeruginosa*. *J. Bacteriol.*, 172:853–866.
36. Fisher, R.F. and Long, S.R., 1989, DNA footprint analysis of the transcriptional activator proteins NodD1 and NodD3 on the inducible *nod* gene promoters. *J. Bacteriol.*, 171:5492–5502.
37. Gaal, T., Ross, W., Blatter, E.E., Tang, H., Jia, X., Krishnan, V.V., Assa-Munt, N., Ebright, R.H., and Gourse, R.L., 1996, DNA-binding determinants of the α subunit of RNA polymerase: Novel DNA-binding domain architecture. *Genes Dev.*, 10:16–26.
38. Gao, J. and Gussin, G.N., 1991, Activation of the *trpBA* promoter of *Pseudomonas aeruginosa* by TrpI protein *in vitro*. *J. Bacteriol.*, 173:3763–3769.
39. Gao, J. and Gussin, G.N., 1991, Mutations in TrpI binding site II that differentially affect activation of the *trpBA* promoter of *Pseudomonas aeruginosa*. *EMBO J.*, 10: 4137–4144.

40. Giladi, H., Murakami, K., Ishihama, A., and Oppenheim, A.B., 1996, Identification of an UP element within the IHF binding site at the P_{L1} - P_{L2} tandem promoter of bacteriophage λ . *J. Mol. Biol.*, 260:484–491.
41. Goncharoff, P. and Nichols, B.P., 1984, Nucleotide sequence of *Escherichia coli* *pabB* indicates a common evolutionary origin of *p*-aminobenzoate synthetase and anthranilate synthetase. *J. Bacteriol.*, 159:57–62.
42. Gosset, G., Bonner, C.A., and Jensen, R.A., 2001, Microbial origin of plant-type 2-keto-3-deoxy-D-arabino-heptulosonate 7-phosphate synthases, exemplified by the chorismate- and tryptophan-regulated enzyme from *Xanthomonas campestris*. *J. Bacteriol.*, 183:4061–4070.
43. Green, J.M., Merkel, W.K., and Nichols, B.P., 1992, Characterization and sequence of *Escherichia coli* *pabC*, the gene encoding aminodeoxychorismate lyase, a pyridoxal phosphate-containing enzyme. *J. Bacteriol.*, 174:5317–5323.
44. Gussin, G.N., Olson, C., Igarashi, K., and Ishihama, A., 1992, Activation defects caused by mutations in *Escherichia coli* *rpoA* are promoter specific. *J. Bacteriol.*, 174:5156–5160.
45. Henikoff, S., Haugn, G.W., Calvo, J.M., and Wallace, J.C., 1988, A large family of bacterial activator proteins. *Proc. Natl. Acad. Sci. USA*, 85:6602–6606.
46. Ho, Y-S. and Rosenberg, M., 1985, Characterization of a third *cII*-dependent, coordinately regulated promoter on phage lambda involved in lysogenic development. *J. Biol. Chem.*, 260:11838–11844.
47. Hochschild, A., Irwin, N., and Ptashne, M., 1982, Repressor structure and the mechanism of positive control. *Cell*, 32:319–325.
48. Holloway, B.W. and Morgan, A.F., 1986, Genome organization in *Pseudomonas*. *Annu. Rev. Microbiol.*, 40:9–105.
49. Holmgren, E. and Crawford, I.P., 1982, Regulation of tryptophan genes in *Rhizobium leguminosarum*. *J. Bacteriol.*, 149:1135–1137.
50. Hoover, T.R., Santero, E., Porter, S., and Kustu, S., 1990, The integration host factor stimulates interaction of RNA polymerase with NIFA, the transcriptional activator for nitrogen fixation operons. *Cell*, 63:11–22.
51. Huang, J. and Schell, M.A., 1991, *In vivo* interactions of the NahR transcriptional activator with its target sequences. *J. Biol. Chem.*, 268:10830–10838.
52. Hwang, J.J. and Gussin, G.N., 1988, Interactions between *E. coli* RNA polymerase and lambda repressor: Mutations in P_{RM} affect repression of P_R . *J. Mol. Biol.*, 200:735–739.
53. Igarashi, I., Hanamura, A., Makino, K., Aiba, H., Aiba, H., Mizuno, T., Nakata, A., and Ishihama, A., 1991, Functional map of the α subunit of *Escherichia coli* RNA polymerase: Two modes of transcription activation by positive factors. *Proc. Natl. Acad. Sci. USA*, 88:8958–8962.
54. Im, S.W.K., Davidson, H., and Pittard, J., 1971, Phenylalanine and tyrosine biosynthesis in *Escherichia coli* K-12: Mutants derperessed for 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (phe), 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (tyr), chorismate mutase T-prephenate dehydrogenase, and transaminase A. *J. Bacteriol.*, 108:400–409.
55. Ito, J. and Crawford, I.P., 1965, Regulation of the enzymes of the tryptophan pathway in *Escherichia coli*. *Genetics*, 52:1303–1316.
56. Jensen, R.A., 1996, Evolution of metabolic pathways in enteric bacteria. In F.C. Neidhardt (ed.), *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn. pp. 2649–2662. ASM Press, Washington, DC.
57. Jensen, R.A. and Ahmad, S., 1988, Evolution and phylogenetic distribution of the specialized isozymes of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase in superfamily-B prokaryotes. *Microbiol. Sci.*, 5:316–319.
58. Jensen, R.A. and Nasser, D.S., 1968, Comparative regulation of isoenzymic DAHP synthetases in microorganisms. *J. Bacteriol.*, 95:188–196.

59. Johnson, A.D., Meyer, B.J., and Ptashne, M., 1979, Interactions between DNA-bound repressors govern regulation by the λ phage repressor. *Proc. Natl. Acad. Sci. USA*, 76:5061–5065.
60. Kane, J.F., Holmes, W.M., and Jensen, R.A., 1972, Metabolic interlock: The dual function of a floate pathway gene as an extra-chromosomal gene of tryptophan biosynthesis. *J. Biol. Chem.*, 247:1587–1596.
61. Kaplan, J.B. and Nichols, B.P., 1983, Nucleotide sequence of *Escherichia coli pabA* and its evolutionary relationship to *trp(G)D*. *J. Mol. Biol.*, 168:451–468.
62. Kishan, V. and Hillen, W., 1990, Molecular cloning, nucleotide sequence, and promoter structure of the *Acinetobacter calcoaceticus trpFB* operon. *J. Bacteriol.*, 172:6151–6155.
63. Kotik, E.A., Olekhovich, I.N., and Fomichev, Y.K., 1996, Regulation of tryptophan biosynthesis in *Pseudomonas mendocina* and *Pseudomonas marginata* bacteria. *Genetika*, 32:1051–1055.
64. Kuldell, N. and Hochschild, A., 1994, Amino acid substitutions in the -35 recognition motif of σ^{70} that result in defects in phage λ repressor-stimulated transcription. *J. Bacteriol.*, 176:2991–2998.
65. Kumamoto, A.A., Miller, W.G., and Gunsalus, R.P., 1987, *Escherichia coli* tryptophan repressor binds multiple sites within the *aroH* and *trp* operators. *Genes Devel.*, 1:556–564.
66. Landick, R. and Yanofsky, C., 1987, Transcription Attenuation. In F.C. Neidhardt, J.L. Ingraham, K.B. Low, B. Magasanik, M. Schaechter, and H.E. Umbarger (eds), *Escherichia coli and Salmonella typhimurium*, vol. 2, pp. 1276–1301. ASM Press, Washington, DC.
67. Landick, R., Turnbough, C.L., Jr, and Yanofsky, C., 1996, Transcription Attenuation, In F.C. Neidhardt (ed.), *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, pp. 1263–1286. ASM Press, Washington DC.
68. Li, M., Moyle, H., and Susskind, M.M., 1994, Target of the transcriptional activation function of phage lambda cI protein. *Science*, 263:75–77.
69. Macchi, R., Montesissa, L., Murakami, K., Ishihama, A., de Lorenzo, V., and Bertoni, G., 2003, Recruitment of σ^{54} -RNA polymerase to the *Pu* promoter of *Pseudomonas putida* through integration host factor-mediated positioning switch of α subunit carboxyl-terminal domain on an UP-like element. *J. Biol. Chem.*, 278:27695–27702.
70. Maksimova, N.P., Olekhovich, I.N., and Fomichev, Y.K., 1991, Regulation of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase synthesis in *Pseudomonas* bacteria. *Genetika*, 27:217–221.
71. Manch, J.N. and Crawford, I.P., 1982, Genetic evidence for a positive regulatory factor mediating induction in the tryptophan pathway of *Pseudomonas aeruginosa*. *J. Mol. Biol.*, 156:67–77.
72. Martin, K., Huo, L., and Schleif, R.F., 1986, The DNA loop model for *ara* repression: AraC protein occupies the proposed loop sites *in vivo* and repression-negative mutations lie in the same site. *Proc. Natl. Acad. Sci. USA*, 83:3654–3658.
73. Matsumoto, H., Furihata, K., Ohnishi, M., and Holloway, B.W., 1995, Clustering of the *trp* genes in *Burkholderia* (formerly *Pseudomonas*) *cepacia*. *FEMS Microbiol. Lett.*, 134:265–271.
74. Maurer, R. and Crawford, I.P., 1971, New regulatory mutation affecting some of the tryptophan genes in *Pseudomonas putida*. *J. Bacteriol.*, 106:331–338.
75. Mavrodi, D.M., Ksenzenko, V.N., Bonsall, R.F., Cook, R.J., Boronin, A.M., and Thomashow, L.S., 1998, A seven-gene locus for synthesis of phenazine-1-carboxylic acid by *Pseudomonas fluorescens* 2–79. *J. Bacteriol.*, 180:2541–2548.
76. Mavrodi, D.M., Bonsall, R.F., Delaney, S.M., Soule, M.J., Phillips, G., and Thomashow, L.S., 2001, Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PAO1. *J. Bacteriol.*, 183:6454–6465.

77. Miles, E.W., Bauerle, R., and Ahmed, S.A., 1987, Tryptophan synthase. *Meth. Enzymol.*, 142:398–414.
78. Miller, A., Wood, D., Ebright, R.H., and Rothman-Denes, L.B., 1997, RNA polymerase β' subunit: A target of DNA binding-independent activation. *Science*, 275:1614–1616.
79. Morollo, A.A. and Bauerle, R., 1993, Characterization of composite aminodeoxyisochorismate synthase and aminodeoxyisochorismate lyase activities of anthranilate synthase. *Proc. Natl. Acad. Sci. USA*, 90:9983–9987.
80. Morollo, A.A. and Eck, M.J., 2001, Structure of the cooperative allosteric anthranilate synthase from *Salmonella typhimurium*. *Nat. Struct. Biol.*, 8:243–247.
81. Nelson, K., Paulsen, I., Weinle, C., Dodson, R., Hilbert, H., Fouts, D., Gill, S., Pop, M., Martins Dos Santos, V., Holmes, M., Brinkac, L., Beanan, M., DeBoy, R., Daugherty, S., Kolonay, J., Madupu, R., Nelson, W., White, O., Peterson, J., Khouri, H., Hance, I., Lee, P., Holtzapple, E., Scanlan, D., Tran, K., Moazzez, A., Utterback, T., Rizzo, M., Lee, K., Kosack, D., Moestl, D., Wedler, H., Lauber, J., Hoheisel, J., Straetz, M., Heim, S., Kiewitz, C., Eisen, J., Timmis, K., Duesterhoft, A., Tummeler, B., and Fraser, C., 2002, Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ. Microbiol.*, 4:799–808.
82. Nichols, B.P., 1996, Evolution of genes and enzymes of tryptophan biosynthesis. In F.C. Neidhardt (ed.), *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn. pp. 2638–2648. ASM Press, Washington, DC.
83. Nichols, B.P., Seibold, A.M., Doktor, S.Z., 1989, Para-aminobenzoate synthesis from chorismate occurs in two steps. *J. Biol. Chem.*, 264:8597–8601.
84. Nickels, B.E., Dove, S.L., Murakami, K.S., Darst, S.A., and Hochschild, A., 2002, Protein–protein and protein–DNA interactions of σ^{70} region 4 involved in transcription activation by λ cI. *J. Mol. Biol.*, 324:17–34.
85. Olekhovich, I.N. and Fomichev, Y.K., 1990, Cloning of the genes of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase of methylotrophic *Pseudomonas* in *Escherichia coli* cells. *Genetika*, 26:418–423.
86. Olekhovich, I. and Gussin, G.N., 1998, Recognition of binding sites I and II by the TrpI activator protein of *Pseudomonas aeruginosa*: Efficient binding to both sites requires InGP even when site II is replaced by site I. *Gene*, 223:247–255.
87. Olekhovich, I. and Gussin, G.N., 2001, Effects of mutations in the *Pseudomonas putida miaA* gene: Regulation of the *trpE* and *trpGDC* operons in *P. putida* by attenuation. *J. Bacteriol.*, 183:3256–3260.
88. Olekhovich, I.N., Maksimova, N.P., and Fomichev, Y.K., 1985, Tryptophan operon of facultative methylotrophic *Pseudomonas* sp. M bacteria. *Genetika*, 21:1627–1633.
89. Olekhovich, I.N., Maksimova, N.P., and Fomichev, Y.K., 1986, Repression of synthesis and retroinhibition of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase operon of facultative methylotrophic *Pseudomonas* sp. M. *Mol. Genet. Mikrobiol. Virusol.*, 12:34–36.
90. Palleroni, N.J., Kunizawa, R., Contopoulou, R., and Doudoroff, M., 1973, Nucleic acid homologies in the genus *Pseudomonas*. *Int. J. Syst. Bacteriol.*, 23:333–339.
91. Pan, P., Woehl, E., and Dunn, M.F., 1997, Protein architecture, dynamics, and allostery in tryptophan synthase channeling. *Trends Biochem. Sci.*, 22:22–27.
92. Panina, E.M., Vitreschak, A.G., Mironov, A.A., and Gelfand, M.S., 2001, Regulation of aromatic amino acid biosynthesis in gamma-proteobacteria. *J. Mol. Microbiol. Biotechnol.*, 3:529–543.
93. Parekh, B.S. and Hatfield, G.W., 1996., Transcriptional activation by protein-induced DNA bending: Evidence for a DNA structural transmission model. *Proc. Natl. Acad. Sci. USA*, 93:1173–1177.
94. Parsons, J.F., Jensen, P.Y., Pachikara, A.S., Howard, A.J., Eisenstein, E., and Ladner, J.E., 2002, Structure of *Escherichia coli* amidodeoxychorismate synthase: Architectural conservation and diversity in chorismate-utilizing enzymes. *Biochemistry*, 41:2198–2208.

95. Perez-Martin, J., Timmis, K.N., and de Lorenzo, V., 1994, Co-regulation by bent DNA. Functional substitutions of the integration host factor site at σ^{54} -dependent promoter *Pu* of the upper-TOL operon by intrinsically curved sequences. *J. Biol. Chem.*, 269:22657–22662.
96. Perez-Martin, J. and Espinosa, M., 1994, Correlation between DNA bending and transcriptional activation at a plasmid promoter. *J. Mol. Biol.*, 241:7–17.
97. Pierson, III, L.S., Keppenne, B.D., and Wood, D.W., 1994, Phenazine antibiotic biosynthesis in *Pseudomonas aureofaciens* 30-84 is regulated by PhzR in response to cell density. *J. Bacteriol.*, 176:3966–3974.
98. Pineiro, S., Olekhnovich, I., and Gussin, G.N., 1997, DNA bending by the TrpI protein of *Pseudomonas aeruginosa*. *J. Bacteriol.*, 179:5407–5413.
99. Pittard, J., Camakaris, L., and Wallace, B.J., 1969, Inhibition of 3-deoxy-D-arabinoheptulosonic acid 7-phosphate synthetase (*trp*) in *Escherichia coli*. *J. Bacteriol.*, 97:1241–1247.
100. Queener, S.W., Queener, S.F., Meeks, J.R., and Gunsalus, I.C., 1973, Anthranilate synthase from *Pseudomonas putida*: Purification and properties of a two-component enzyme. *J. Biol. Chem.*, 248:151–161.
101. Queener, S.W. and Gunsalus, I.C., 1970, Anthranilate synthase enzyme system and complementation in *Pseudomonas*. *Proc. Natl. Acad. Sci. USA*, 67:1225–1232.
102. Ren, Y.-L., Garges, S., Adhya, S., and Krakow, J.S., 1988, Cooperative DNA binding of heterologous proteins: Evidence for contact between the cyclic AMP receptor protein and RNA polymerase. *Proc. Natl. Acad. Sci. USA*, 85:4138–4142.
103. Ross, W., Gosink, K.K., Salomon, J.K.L., Zou, C., Ishihama, A., Severinov, K., and Gourse, R.L., 1993, A third recognition element in bacterial promoters: DNA binding by the α subunit of RNA polymerase. *Science*, 262:1407–1413.
104. Sawula, R.V. and Crawford, I.P., 1972, Mapping of the tryptophan genes of *Acinetobacter calcoaceticus* by transformation. *J. Bacteriol.*, 112:797–805.
105. Schell, M.A., 1993, Molecular biology of the LysR family of transcriptional regulators. *Annu. Rev. Microbiol.*, 47:597–626.
106. Schell, M.A., Brown, P.H., and Mol, S.R., 1990, Use of saturation mutagenesis to localize probable functional domains in the NahR Protein, a LysR-type transcription activator. *J. Biol. Chem.*, 265:3844–3850.
107. Song, J. and Jensen, R.A., 1996, PhhR, a divergently transcribed activator of the phenylalanine hydroxylase gene cluster of *Pseudomonas aeruginosa*. *Mol. Microbiol.*, 22:497–507.
108. Spraggon, G., Kim, C., Xuong, N.-H., Yee, M.-C., Yanofsky, C., and Mills, S.E., 2001, The structures of anthranilate synthase of *Serratia marcescens* crystallized in the presence of (i) its substrates, chorismate and glutamine, and a product, glutamate, and (ii) its end-product inhibitor, L-tryptophan. *Proc. Natl. Acad. Sci. USA*, 98:6021–6026.
109. Storz, G., Tartaglia, L.A., and Ames, B.N., 1990, Transcriptional regulator of oxidative stress-inducible genes: Direct activation by oxidation. *Science*, 248:189–194.
110. Stover, C.K., Pham, X.-Q.T., Erwin, A.L., Mizoguchi, S.D., Warriner, P., Hickey, M.J., Brinkman, F.S.L., Hufnagle, W.O., Kowalik, D.J., Lagrou, M., Garber, R.L., Goltry, L., Tolentino, E., Westbrook-Wadman, S., Yuan, Y., Brody, L.L., Coulter, S.N., Folger, K.R., Kas, A., Larbig, K., Lim, R.M., Smith, K.A., Spencer, D.H., Wong, G.K.-S., Wu, Z., Paulsen, I.T., Reizer, J., Saier, M.H., Hancock, R.E.W., Lory, S. and Olson, M.V., 2000, Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature*, 406: 959–964.
111. Subramaniam, P.S., Xie, G., Xia, T., and Jensen, R.A., 1998, Substrate ambiguity of 3-deoxy-D-manno-octulosonate 8-phosphate synthase from *Neisseria gonorrhoeae* in the context of its membership in a protein family containing a subset of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthases. *J. Bacteriol.*, 180:119–127.
112. Tan, S. and Richmond, T.J., 1990, DNA binding-induced conformational change of the yeast transcriptional activator PRTF. *Cell*, 67:367–377.

113. Urbanowski, M.L. and Stauffer, G.V., 1989, Role of homocysteine in *metR*-mediated activation of the *metE* and *metH* genes in *Salmonella typhimurium* and *E. coli*. *J. Bacteriol.*, 171:3277–3281.
114. Wek, R.C. and Hatfield, G.W., 1988, Transcriptional activation at adjacent operators in the divergent-overlapping *ilvY* and *ilvC* promoters of *Escherichia coli*. *J. Mol. Biol.*, 203:643–663.
115. Whitaker, R.J., Berry, A., Byng, G.S., Fiske, M.J., and Jensen, R.A., 1985, Clues from *Xanthomonas campestris* about the evolution of aromatic biosynthesis and its regulation. *J. Mol. Evol.*, 21:139–149.
116. Whitaker, R.J., Fiske, M.J., and Jensen, R.A., 1982, *Pseudomonas aeruginosa* possesses two novel regulatory isozymes of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase. *J. Biol. Chem.*, 257:12789–12794.
117. Whitely, M., Lee, K.M., and Greenberg, E.P., 1999, Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA*, 96:13904–13909.
118. Yanofsky, C., 1984, Comparison of regulatory and structural regions of genes of tryptophan metabolism. *Mol. Biol. Evol.*, 1:143–161.
119. Yanofsky, C. and Crawford, I.P., 1987, The Tryptophan Operon. In F.C. Neidhardt, J.L. Ingraham, K.B. Low, B. Magasanik, M. Schaechter, and H.E. Umbarger (eds), *Escherichia coli and Salmonella typhimurium*, vol. 2, pp. 1453–1472. ASM Press, Washington, DC.
120. Yanofsky, C., Kelley, R.L., and Horn, V., 1984, Repression is relieved before attenuation in the *trp* operon of *Escherichia coli* as tryptophan starvation becomes increasingly severe. *J. Bacteriol.*, 158:1018–1024.
121. Yanofsky, C. and Soll, L., 1977, Mutations affecting tRNA^{trp} and its charging and their effect on regulation of transcription termination at the attenuator of the tryptophan operon. *J. Mol. Biol.*, 113:663–677.
122. Zalkin, H., 1980, Anthranilate synthase: Relationships between bifunctional and monofunctional enzymes. In H. Bisswanger and E. Schminke-Ott (eds), *Multifunctional Proteins*, pp. 123–149. Wiley, New York.
123. Zalkin, H., 1993, The amidotransferases. *Adv. Enzymol. Relat. Areas Mol. Biol.* 66:203–309.
124. Zalkin, H. and Hwang, L.H., 1971, Anthranilate synthase from *Serratia marcescens*: On the properties and relationship to the enzyme from *Salmonella typhimurium*. *J. Biol. Chem.*, 246:6899–6907.
125. Zhou, Y., Zhang, X., and Ebright, R., 1993, Identification of the activating region of catabolite gene activator protein (CAP): Isolation and characterization of mutants of CAP specifically defective in transcription activation. *Proc. Natl. Acad. Sci. USA*, 90:6081–6085.
126. Zurawski, G., Gunsalus, R.P., Brown, K.D., and Yanofsky, C., 1981, Structure and regulation of *aroH*, the structural gene for the tryptophan-repressible 3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase of *Escherichia coli*. *J. Mol. Biol.*, 145:47–73.

METABOLISM OF SULPHUR-CONTAINING ORGANIC COMPOUNDS

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1. INTRODUCTION

Pseudomonas species inhabit a wide variety of habitats, ranging from the human body to soils, the rhizosphere, and the phyllosphere. Like all microorganisms, they require sulfur for growth, and this is normally provided by assimilation of inorganic sulfate. However, many pseudomonads inhabit environments where sulfate may not be freely available. In aerobic soils, for instance, inorganic sulfate makes up less than 5% of the total sulfur, and most of the residual sulfur is present as peptides/amino acids, sulfate esters, and sulfonates. Much of this sulfonate content is derived from plant sulfolipid in the thylakoid membranes, which may also provide sulfur for leaf-dwelling pseudomonads. In the human body, other sulfur sources are present, such as the neurotransmitter taurine (2-aminoethanesulfonate), sulfated mucins in gut and lung environments, and glycosaminoglycan in connective tissue. The importance of sulfur metabolism for pseudomonads is underlined by the fact that in *Pseudomonas syringae* methionine prototrophy is required for virulence⁸, while in *Pseudomonas putida* loss of the ability to desulfurize sulfate esters leads to reduced survival in the soil⁵⁴.

Many sulfur-containing compounds are xenobiotic in nature, including numerous surfactants and dyestuffs. Biodegradation studies have reported many *Pseudomonas* strains that are able to transform these compounds. However, the breadth of the original *Pseudomonas* classification has meant that many of these strains are now assigned to different taxonomic divisions, particularly *Comamonas*, *Sphingomonas*, and *Ralstonia*. Since many desulfurization mechanisms have been elucidated in these strains, and the relevant biochemical pathways are often also present in *Pseudomonas* species, they are included here where necessary.

Wherever possible, this chapter links biochemical data available in the literature to the *Pseudomonas* genomic data available at the time of writing (annotated genome sequences for *Pseudomonas aeruginosa* PAO1¹¹¹, *P. putida* KT2440⁸² and *P. syringae* DC3000 [TIGR] and sequence data for *Pseudomonas fluorescens* SBW-25 [Wellcome Trust Sanger Institute], for which sequencing is essentially complete, but annotation has not been finished). Comparison of these genome sequences with genetic data from other strains has revealed the flexibility of *Pseudomonas* strains, and their genetic plasticity. Expression of genes related to organosulfur metabolism is upregulated under low-sulfur conditions^{91, 92}, and the encoded proteins hence often contain lower than usual levels of the sulfur-containing amino acids cysteine and methionine. The genes for such proteins are clustered in the *Pseudomonas* genomes, as has previously been observed for *Escherichia coli*⁹⁸. The clearest example is in the *P. putida* KT2440 genome, where the genes for taurine and alkanesulfonate metabolism are clustered together with those for cysteine transport, glutathione synthesis and uncharacterized genes related to dibenzothiophene (DBT) desulfurization. This clustering has not been explored in detail as yet, but is an interesting facet of microbial sulfur metabolism that deserves increased attention.

Desulfurization of organosulfur compounds often represents the first step in mineralization of those compounds, but by releasing inorganic sulfur it also constitutes the initial step in cysteine biosynthesis. This chapter will present an overview of the cysteine and methionine biosynthetic pathways, concentrating on how the *Pseudomonas* pathways differ from those known in other bacteria. The emphasis in the following sections will then be on desulfurization of organosulfur compounds, and how it is regulated in pseudomonads.

2. CYSTEINE AND METHIONINE METABOLISM

2.1. Cysteine Biosynthesis in Pseudomonads

The biosynthesis of cysteine from inorganic sulfate has been extensively studied both in eukaryotes and in microorganisms. In bacteria, the pathway has

been best characterized for *E. coli*⁶⁷, and many steps in the *E. coli* pathway are very similar to those seen for pseudomonads (Figure 1). Assimilation of inorganic sulfate proceeds by transport of inorganic sulfate into the cell by an ABC-type transporter consisting of a periplasmic binding protein Sbp, permease components CysT and CysW, and an ATPase component CysA—thiosulfate uptake is catalysed by interaction of the same membrane components with the CysP binding protein. Sulfate is activated first to adenosine-5'-phosphosulfate (APS) and then to phosphoadenosine-5'-phosphosulfate (PAPS) by ATP sulfurylase and APS kinase. PAPS is then reduced by a thioredoxin-dependent PAPS reductase to yield sulfite, which is further reduced to sulfide by sulfite reductase. This sulfide is then transferred onto the carbon backbone of serine by the cysteine synthase complex, generating cysteine. In enteric bacteria, methionine is synthesized by transsulfuration of cysteine to homocysteine, followed by methylation to afford methionine.

Cysteine and methionine biosynthesis in pseudomonads differs from the paradigm presented in enteric bacteria in a number of important ways, and the overall pathway is shown in Figure 1. Analysis of the *P. aeruginosa*, *P. putida*, and *P. syringae* genome sequences reveals the presence of an ABC-type transporter for sulfate/thiosulfate in all three species (*sbp-cysTWA*, with an unlinked *cysP* gene in *P. aeruginosa* and *P. syringae*, whereas *P. putida* contains *cysPTWA*, and an unlinked *sbp* gene). Expression of *sbp* has been shown to be upregulated under sulfate-limited conditions in *P. aeruginosa*⁹¹, and it therefore seems likely that sulfate uptake in this species is similar to *E. coli*. However, in *P. aeruginosa*, several putative plant-type sulfate transporters of

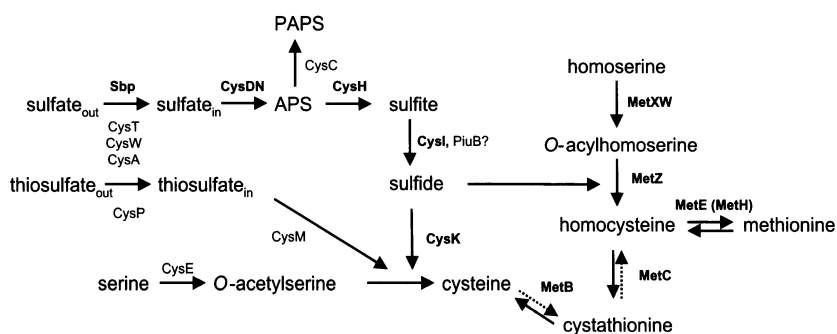


Figure 1. Cysteine biosynthesis in pseudomonads. Gene products shown in bold type have been demonstrated to be active in *Pseudomonas* species. The remaining gene products are present in *Pseudomonas* genome sequences, and have been assigned by comparison to other species. Cysteine conversion to homoserine via cystathionine (dotted arrows) occurs only when cysteine is supplied as sulfur source¹³⁰. Sulfur from sulfate esters enters the pathway at the level of intracellular sulfate, whereas sulfur from sulfonates is assimilated as sulfite.

the SulP family^{57, 88} are also present in the genome. These permeases are members of the major facilitator superfamily, and act as sulfate:proton symporters, but it is not known if they are expressed or active in *P. aeruginosa*, nor in *P. fluorescens* and *P. syringae*, where homologues are also present.

Activation of sulfate to APS requires the *cysD* and *cysN* genes, encoding the two subunits of ATP sulfurylase. In all four *Pseudomonas* genomes that have been sequenced to date, the *cysD* and *cysN* genes form a putative operon, and the *cysN* gene is fused to a terminal domain with high similarity to the *cysC* gene of other bacteria. Since *cysC* encodes APS kinase, this would suggest that sulfate is rapidly converted into PAPS in pseudomonads. However, two lines of evidence oppose this. Firstly, the *P. aeruginosa cysN* gene will not restore growth to an *E. coli cysC* mutant, suggesting that the C-terminal, *cysC*-like domain of *P. aeruginosa* CysN is not active as an APS kinase (M. Kertesz, unpublished results). Secondly, *P. aeruginosa* also contains a separate, single-domain CysC protein, which contains the conserved Ser-105 which is phosphorylated as part of the catalytic mechanism in *E. coli* CysC¹⁰⁰. Interestingly, the fused CysNC proteins from the *Pseudomonas* genome sequences all lack this active site serine (changed to an alanine), suggesting that the *cysC*-like domain may be inactive in all cases. Mutant studies have confirmed that *P. aeruginosa cysN* is required for sulfate assimilation⁴⁶, so the N-terminal portion is presumably active in sulfate activation.

In fact, a functional APS kinase (CysC) is not required for cysteine biosynthesis in pseudomonads, since *P. aeruginosa cysH* does not encode a PAPS reductase, as in *E. coli*, but an APS reductase¹⁰, and PAPS is therefore entirely bypassed in the cysteine biosynthetic pathway. The specificity of the purified *P. aeruginosa* CysH protein as an APS reductase but not a PAPS reductase appears to be due to the presence of a [4Fe-4S] cluster that is absent in the PAPS-reducing enzyme from *E. coli*⁶⁶. Unlike plant APS reductases, *P. aeruginosa* CysH does not possess a thioredoxin-like domain, and the enzyme requires added thioredoxin for maximum activity⁶⁶. Phylogenetic analysis showed the presence of an APS reductase in a variety of pseudomonads (*Pseudomonas*, *Burkholderia*, *Ralstonia*, *Sphingomonas*), and the PAPS reduction pathway seems to be confined to enterobacteria, yeasts and fungi, and some cyanobacteria⁶⁶. PAPS may still be required by the cell as a sulfur donor in sulfation reactions, and this is reflected in the presence of a *cysC* gene in *P. aeruginosa* and *P. syringae* (although this gene appears to be absent from the *P. putida* genome).

Reduction of APS yields sulfite, and this is further reduced to sulfide by a sulfite reductase. In pseudomonads, this step is probably catalysed by a similar enzyme to the NADPH-dependent sulfite reductase that is found in enterobacteria, consisting of flavoprotein (CysJ) and heme-protein (CysI) subunits. The *cysI* gene has been shown to be required for the growth of

P. aeruginosa with either sulfate or sulfite as sulfur source, confirming its importance in sulfite reduction⁴⁶. However, the only *cysJ* homologue in this species is the C-terminal domain of an iron-regulated gene *piuB*⁸³, and though this link between cysteine biosynthesis and iron supply is a fascinating one, it needs to be confirmed at a biochemical level. The *piuB-cysJ* fusion is conserved in all four sequenced *Pseudomonas* genomes. *cysI* and *piuB-cysJ* are located separately from each other in *P. putida* and *P. aeruginosa*, unlike the situation in *E. coli* where *cysJIH* constitutes a single transcriptional unit. Sulfite reductase is an important enzyme in assimilation of organosulfur compounds, since the sulfur moiety of sulfonates and DMSO is released as sulfite, which can then be further assimilated to cysteine or methionine.

Synthesis of cysteine from *O*-acetylserine and sulfide has not been studied in any detail in pseudomonads. In *E. coli*, this step is catalyzed by two pyridoxal phosphate-dependent isozymes, designated *O*-acetylserine sulfhydrylase A and B, and encoded by the *cysK* and *cysM* genes, respectively. Each of the sequenced *Pseudomonas* genomes contains homologues of both of these isozymes, and it seems likely that their biochemistry is similar to that reported in enterobacteria.

In *P. aeruginosa* and in *P. putida*, cysteine is synthesized from sulfide by sulfhydrylation of acetylserine, as in enteric bacteria¹³⁰. However, the sulfide generated by sulfate assimilation is also used for the direct synthesis of homocysteine by an *O*-acylhomoserine sulfhydrylase³⁹. Measurements of the acetylserine- and succinylhomoserine sulfhydrylases in cell extracts of *P. aeruginosa* and *P. putida* show that the two enzymes were present at similar levels of activity¹³⁰ during growth with organosulfur compounds, but sulfhydrylation of *O*-succinylhomoserine was reduced by 50% during growth of either of the above species with inorganic sulfate. Direct sulfhydrylation of succinylhomoserine then leads to methionine in two further steps.

2.2. Methionine Biosynthesis in Pseudomonads

The ability to synthesize methionine is essential for protein biosynthesis, but in several pseudomonads it is also required for virulence and pathogenesis, and in *P. syringae* methionine auxotrophs show reduced survival in the phyllosphere^{2, 7, 8}. The reason for this is unclear, since leaf exudates are known to contain methionine, and methionine concentrations are not growth limiting on leaf surfaces⁸. As indicated above, synthesis of methionine by pseudomonads does not normally proceed via cysteine, but is initiated by direct sulfhydrylation of an *O*-acylhomoserine derivative. In this respect, *Pseudomonas* species are more similar to fungi and Gram-positive bacteria than they are to other Gram-negative species. The nature of the *O*-acylhomoserine derivative varies between species. In *P. syringae* *O*-acetylhomoserine is the

sulphydrylation substrate, and it is generated by the *metW* and *metX* gene products². MetX shows homology to homoserine *O*-acetyltransferases of various species, and homologues are found in all the *Pseudomonas* genomes that have been sequenced. However, the substrate specificity of this enzyme appears to vary between species, since in *P. aeruginosa* *O*-acetylhomoserine is not a substrate for subsequent sulphydrylation³⁹, which requires the *O*-succinyl derivative. It should be pointed out that although Foglino *et al.* assumed that the succinylhomoserine synthase activity was catalyzed by a protein similar to *E. coli* MetA, in fact the gene responsible (mapped at 20' on the PAO1 chromosome³⁹, and required for methionine prototrophy) corresponds to the *metXW* locus of other pseudomonads. *metW* is well conserved in a number of species and is required for methionine prototrophy², but its function has not yet been clearly elucidated.

Sulphydrylation to yield homocysteine is catalyzed by the *metZ* gene product, which is conserved in *P. aeruginosa*, *P. putida*, and *P. fluorescens*. *metZ* encodes the *O*-acylhomoserine sulphydrylase, and *metZ* mutants of *P. aeruginosa*³⁹ and *P. putida*¹ are methionine auxotrophs. However, the *P. aeruginosa* *metZ* mutant was able to grow slowly with cysteine³⁹, suggesting that the alternative pathway of cysteine biosynthesis, via transsulfuration to cystathionine, is active in this species. This was confirmed by enzyme measurements in both *P. putida* and *P. aeruginosa*, which showed that cystathionine β -lyase is strongly upregulated in both these species during growth with cysteine as the sole sulfur source¹³⁰.

Methylation of homocysteine to yield methionine is mediated in many microorganisms by two independent methionine synthetases, one of which is cobalamin-dependent (MetH), and the other not (MetE). The *metH* gene is present in all the sequenced *Pseudomonas* genomes, and is highly conserved, and a cobalamin-dependent methionine synthase activity has been reported in *P. aeruginosa*¹³⁴. *P. putida* mutants in *metE* and *metH* genes were methionine prototrophs but the double mutant required exogenous methionine for growth, confirming that both pathways are active in this species¹.

2.3. Methionine Catabolism—Reverse Transsulfurylation or Cleavage?

All four sequenced *Pseudomonas* species grow well when methionine is provided as the sole sulfur source (Table 1), implying that they are able to convert methionine to cysteine efficiently. The two known metabolic pathways for this conversion are (a) desulfurization of methionine to yield methanethiol, and re-assimilation of the sulfur, and (b) reverse transsulfuration from methionine to homocysteine and hence to cysteine via cystathionine. The presence of

Table 1. Relative generation times of *Pseudomonas* species with different sole sulfur sources^a.

Sulfur source (500 μ M)	Relative doubling time			
	<i>P. aeruginosa</i> PAO1	<i>P. fluorescens</i> SBW25	<i>P. putida</i> KT2440	<i>P. syringae</i> DC3000
Sulfate	100	100	100	100
Thiosulfate	90	108	96	101
Methionine	120	110	114	152
Dimethylsulfoxide	129	167	117	146
Dimethylsulfone	134	211	125	206
KSCN	87	—	102	—
Methyl sulfate	98	335	251	88
Hexyl sulfate	107	114	—	411
Dodecyl sulfate	87	193	—	—
Nitrophenylsulfate	109	116	112	105
Nitrocatechol sulfate	104	124	413	—
X-sulfate	129	126	—	—
Cyclohexylsulfamate	121	—	289	—
Pig mucin	742	—	720	—
Methanesulfonate	104	107	99	112
Ethanesulfonate	112	115	103	137
Pentanesulfonate	129	119	122	186
Dodecanesulfonate	—	167	—	—
Taurine	100	122	100	145
Isethionate	109	103	104	117
Cysteate	—	176	333	220
Coenzyme M	115	139	120	128
MOPS	121	113	121	195
PIPES	143	207	227	—
Cysteine	92	137	116	223
Djenkolic acid	—	160	233	356
Glutathione	—	—	—	—
Homocysteine	109	130	106	—
Dithiothreitol	—	516	487	—
Thioglycerol	—	200	—	—
S-Methyl cysteine	125	190	122	360
Thioglucose	92	140	96	112

Doubling times for each species are normalized relative to the generation time with sulfate as sulfur source.

^aCells were cultivated with continuous shaking in a microwell plate with 200 μ L of a succinate-based minimal medium per well ^{9,61}. The sulfur sources listed were provided at 500 μ M, and the experiment was conducted in a Synergy HT microplate reader (Bio-Tek) with KC4 software. Growth temperatures were 37°C (*P. aeruginosa*), 30°C (*P. putida*) or 25°C (*P. fluorescens*, *P. syringae*), as appropriate. Generation times were determined for each strain in 4–8 independent experiments, and all standard deviations are <10% of the mean. Since the strains differ in growth rate, the values given are normalized to the generation time with sulfate, which under these growth conditions was: *P. aeruginosa* 99 \pm 9 min; *P. putida* 77 \pm 3 min; *P. fluorescens* 114 \pm 8 min; *P. syringae* 182 \pm 6 min.

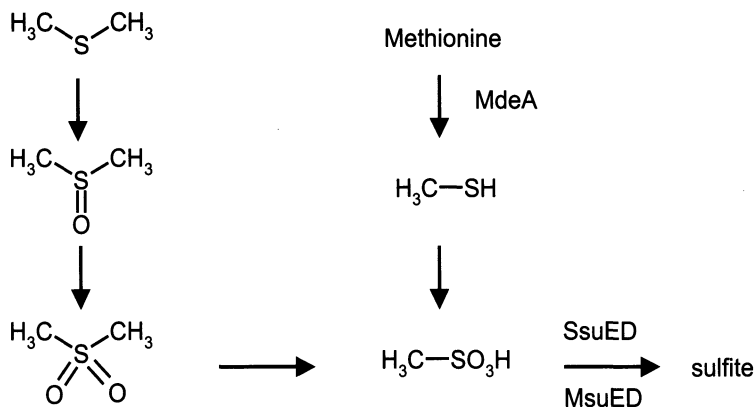


Figure 2. Desulfurization of dimethylsulfide and methionine in *P. putida*. Conversion of dimethylsulfide to methanesulfonate via dimethylsulfone has been demonstrated in *P. putida* DS1³⁴, though the genes responsible are unknown. Cleavage of methionine yields methanethiol⁴⁹, and indirect evidence indicates that this thiol is also assimilated via methanesulfonate¹³⁰.

the latter pathway is characterized phenotypically by the fact that mutant strains unable to assimilate sulfate may be rescued by methionine in the growth medium, and this is indeed observed in *P. aeruginosa*⁴² and in *P. syringae*². In *P. putida* S-313 cells grew normally with methionine even in the presence of selenate, which inhibits sulfate assimilation¹³⁰, suggesting that the reverse transulfuration pathway was also active in this species. However, enzyme measurements revealed that the key enzyme in the pathway, cystathionine- γ -lyase, was inactive during growth with methionine, and that methionine- γ -lyase was strongly upregulated under these conditions. Conversion of methionine to cysteine therefore appears to proceed via cleavage of methionine to methanethiol, oxidation to methanesulfonate, and oxygenolytic cleavage to release sulfite¹³⁰ (Figure 2). *P. putida* methionine lyase is encoded by the *mdeA* gene, which in *P. putida* ICR3460 is co-transcribed with *mdeB*⁵⁰. The *mdeB* gene product is involved in further metabolism of the α -ketobutyrate produced on dethiomethylation of methionine⁴⁹. Expression of *mdeAB* is controlled by a divergently encoded positive regulator, MdeR, belonging to the Lrp family⁴⁹. In *P. putida* KT2440, the *mdeB* gene is absent, and its role is presumably played by another dehydrogenase.

3. SULFONATES

In nature, sulfur is available for bacterial growth in a variety of different forms, and not only as inorganic sulfate. *Pseudomonas* species are very

flexible in their ability to use organic sulfur, and can desulfurize many different types of molecules. Organosulfur compounds can be subdivided into those where the sulfur is directly linked to the carbon chain and those where the linkage is via an oxygen or nitrogen atom. The first form is typified by the sulfonate moiety ($\text{C-SO}_3\text{H}$), whereas sulfate esters ($\text{C-O-SO}_3\text{H}$) or sulfamates ($\text{C-NH-SO}_3\text{H}$) are members of the second group. It should be noted that the former group also includes sulfur-containing heterocycles and heteroaromatics, and these are not well desulfurized by pseudomonads. Table 1 lists relative generation times for the four sequenced *Pseudomonas* species, cultivated in a succinate-minimal medium with a variety of different sulfur sources. For purposes of comparison, the values given are normalized to the growth rates observed with inorganic sulfate. It is immediately apparent that under these conditions, the tested strains are not able to grow well with many reduced sulfur compounds, though cysteine itself is readily accepted as a sulfur source by all except *P. syringae*. *P. aeruginosa* accepts the most diverse range of substrates, including a range of sulfate esters and sulfamates, which are less well desulfurized by *P. fluorescens*, and barely accepted as substrates at all by *P. putida* KT2440 and *P. syringae*. By contrast, the sulfonates are readily desulfurized by all the sequenced *Pseudomonas* species, though the efficiency of utilization varies. This probably reflects the fact that sulfonates make up a large proportion of the sulfur found in soils and sediments^{124, 125}, much of which is probably derived from plant sulfolipid, and that methanesulfonate is the main biogenic sulfur component in the atmosphere⁵.

3.1. Alkanesulfonates

Pseudomonas isolates that are able to degrade the carbon skeleton of simple $\text{C}_4\text{-C}_{12}$ alkanesulfonates have been known for many years^{75, 118}. Biochemical studies on these isolates revealed that the initial step in the mineralization pathway is an oxygen-dependent desulfonation to yield the corresponding aldehyde and sulfite¹¹⁹. The phylogeny of these early isolates was not further investigated, but later studies have repeatedly identified *Delftia* (*Comamonas*) species as degraders of linear and substituted alkanesulfonates^{65, 90, 97}. In *Delftia* (*Comamonas*) *acidovorans* P53, a putative multicomponent monooxygenase responsible for 1-propanesulfonic acid desulfonation has been detected in cell extracts⁹⁶. Oxygenolytic cleavage is also involved in the degradation of the secondary alkanesulfonate sulfosuccinate by *Pseudomonas* sp. B1, with oxygenation occurring at the α -position to the sulfonate group⁹³. However, the carbon-limited desulfonation of aliphatic sulfonates has not been studied in detail in pseudomonads, and although methanesulfonate desulfonation by the methanesulfonate monooxygenase

system (MSAMO) of methylotrophs is well understood, the biochemistry of the two systems is likely to be fundamentally different.

The ability to degrade alkanesulfonates as carbon sources appears to be limited to strains that have been isolated by enrichment for this phenotype. By contrast, alkanesulfonates can be readily utilized as sulfur sources by many pseudomonads. The range of sulfonates used includes natural compounds such as cysteate, taurine and isethionate¹⁰³, but also synthetic alkanesulfonates^{46, 58}, including fluorinated molecules⁶³. Desulfonation leads to the corresponding aldehyde, as observed earlier when sulfonates are utilized as a carbon source¹¹⁹. However, the enzymes concerned are specific for sulfur metabolism, and their synthesis is repressed in the presence of inorganic sulfate (originally observed in *P. aeruginosa* by 2D-PAGE^{46, 91}, and confirmed at transcript level⁹¹).

Desulfonation of alkanesulfonates to provide sulfur for growth has been best studied in a *P. putida* isolate, strain S-313. Desulfonation is catalyzed by the products of the *ssuD* and *ssuE* genes, which encode an FMN H_2 -dependent monooxygenase and an NADPH-dependent FMN reductase, respectively¹²² (Figure 3A). SsuE is quite specific for FMN (K_m 9.1 μM) and NADPH (K_m 0.93 mM), though it will also reduce FAD at a lower rate. SsuD, by contrast, accepts a broad range of sulfonated substrates (K_m [pentanesulfonate]

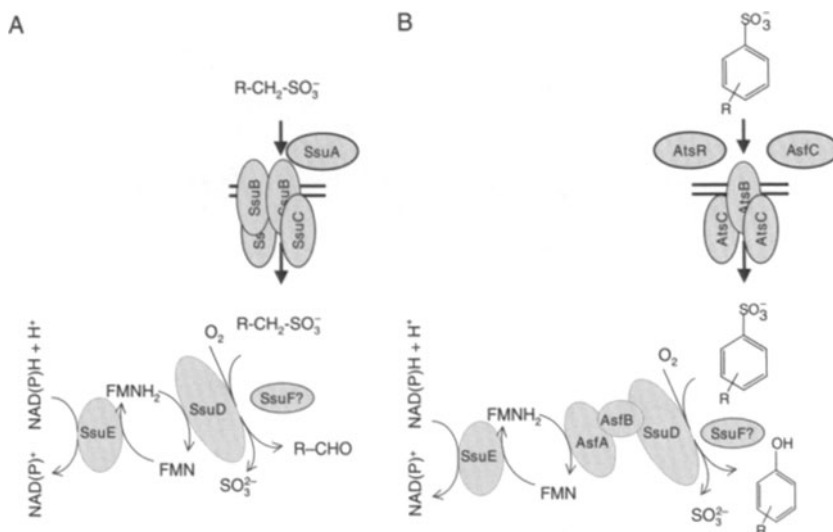
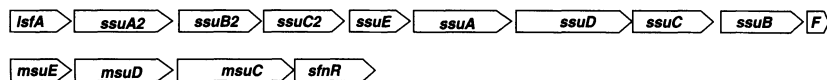


Figure 3. Desulfonation of A. alkylsulfonates and B. arylsulfonates in *P. putida* S-313. The sulfonates are transported into the cell by the SsuABC and AtsR/AsfC-AtsBC complexes, respectively, and desulfonation is catalyzed by the monooxygenase SsuD. See text for details.

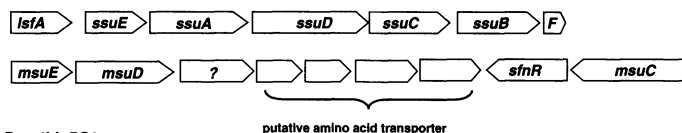
149 μM)¹²². *P. putida* SsuD requires reduced FMN for desulfonating activity in vitro, but this may be provided either by the homologous FMN reductase (SsuE), or by the flavin reductase of *Vibrio fischeri*. It therefore appears that the reductase enzyme does not form an essential part of a desulfonating enzyme complex, but that FMNH₂ may be supplied from the intracellular pool of reduced flavin.

The *ssuE* and *ssuD* genes are contained within a larger operon, *ssuEADCBF*, which also includes genes for an ABC-type transport system (*ssuABC*), and for a putative molybdopterin-binding protein (*ssuF*)⁵⁵ (Figure 4). The sulfonate transporter genes *ssuABC* are not essential for growth of *P. putida* with alkanesulfonates⁵⁵—this agrees with studies carried out in *E. coli* that showed that the sulfonate transporters encoded by *ssuABC* and *tauABC* overlap in substrate specificity³¹. In *P. aeruginosa*, the *ssu* operon is preceded by a second, closely related ABC-type transporter, whose function is unexplored, and by a gene encoding a thiol-specific antioxidant, LsfA. Synthesis of this antioxidant protein is strongly upregulated under sulfate limitation⁹¹, and it may play a role in protecting the cell from oxidative stress caused by overproduction of SsuE under these conditions, and subsequent autoxidation of the FMNH₂ produced. The *lsfA* gene is present in *P. putida*, *P. aeruginosa* and

P. aeruginosa PAO1



P. putida KT2440 and *P. putida* S-313



P. putida DS1



P. syringae DC-3000



Figure 4. Gene loci for cleavage of alkanesulfonates in *Pseudomonas* spp. The *ssu* loci of *P. aeruginosa* (PA3441-PA3450) and *P. putida* (PP0235-PP0241) are similar, with genes for an additional transporter of unknown specificity inserted in the *P. aeruginosa* cluster. The *msu* cluster of *P. putida* KT2440 (PP2764-PP2772) reveals an additional putative transporter compared to that of *P. aeruginosa*. The *sfn* genes of *P. putida* DS1 (acc no. AB091764) are related to the *P. aeruginosa* *msu* genes (PA2354-PP2357), but lack the oxygenase gene *msuD*.

P. syringae but is not associated with *ssu* in *Escherichia coli* or *Bacillus subtilis*. A further difference from other bacterial desulfonation systems is the requirement for the *ssuF* gene product for growth. The *ssuF* gene encodes a putative molybdopterin-binding protein (43% identity to clostridial molybdopterin-binding proteins), and deletion of the *ssuF* gene in *P. putida* S-313 prevents growth with sulfonates, but also with sulfate esters⁵⁵. Neither *E. coli* nor *B. subtilis* have a corresponding gene in the *ssu* operon^{126, 128}, and an *E. coli moaA* mutant which is deficient in molybdopterin synthesis grew normally with pentanesulfonate⁵⁵. The exact role of SsuF in the desulfonation process is therefore still unclear.

The *ssuF* and *ssuD* gene products are also required for growth of another *P. putida* isolate, *P. putida* DS1, with dimethyl sulfide as the sulfur source³⁴. Dimethylsulfide (DMS) is an important biogenic sulfur metabolite that is generated from dimethylsulfoniopropionate in marine systems. In *P. putida* DS1, DMS is oxidized via dimethylsulfoxide and dimethylsulfone to methanesulfonate (Figure 2). Although the enzymes that catalyze the oxidation of DMS to methanesulfonate have not yet been identified, they are likely to be related to those that oxidize DBT to hydroxyphenyl benzenesulfonate in *Rhodococcus* species, DszC and DszA. Cleavage of methanesulfonate in *P. putida* is carried out by the SsuED monooxygenase^{34, 55}.

In *P. aeruginosa*, however, a second, closely related enzyme system exists for cleavage of methanesulfonate. The *msuED* genes encode an FMNH₂-dependent desulfonating monooxygenase system that has enhanced activity for methanesulfonate, though it also desulfonates other alkanesulfonates at a lower rate⁶². The MsuD protein is very similar to SsuD of *P. aeruginosa* (72% identity), *P. putida* (70% identity) and *E. coli* (66% identity), and it is interesting that methanesulfonate desulfonation is catalyzed by the *Pseudomonas* SsuD and MsuD enzymes^{34, 62, 122}, but not by *E. coli* SsuD³⁰. The genetic organization of the *msu* locus is somewhat variable between the *Pseudomonas* isolates (Figure 4). In *P. aeruginosa*, the genes form an operon (*msuEDC*), but in *P. putida* KT2440 *msuC* is absent, and replaced by a putative ABC-type transporter. The putative *msuD* gene of strain KT2440 is not well conserved (28% identity to *P. aeruginosa* MsuD), and indeed, in *P. putida* DS1, it is absent altogether, the corresponding operon consisting of *sfnECR*, where SfnE and SfnC are closely related to MsuE and MsuC, respectively, and SfnR is a σ^{54} -dependent transcriptional activator that is important in dimethylsulfone metabolism (see below). *P. fluorescens* SBW-25 appears to have an *msuEDC* operon similar to *P. aeruginosa*, but its role has not yet been further investigated at a biochemical level. Intriguingly, *P. syringae* has a modified *ssu* cluster consisting only of *lsfA*, the *ssuABC* sulfonate transporter, and *ssuF*. The MsuD and MsuE genes are encoded elsewhere on the chromosome, the former adjacent to an *sfnR* homolog. Although *P. syringae* grows well with methanesulfonate (Table 1), its growth rate with higher sulfonates is reduced compared to other species, probably reflecting the lack of an SsuD protein.

The functions of M_{su}E and M_{su}C have been investigated after over-expression in *E. coli*⁶². M_{su}E shows flavin reductase activity with both NADPH and NADH as the electron donor, contrasting with S_{su}E from both *P. putida* and *E. coli*, which are specific for NADPH. M_{su}C is more of an enigma. It is quite similar (42% identity) to D_{sz}C, the enzyme that carries out the initial stepwise oxidation of DBT to DBT-oxide and DBT-dioxide in *Rhodococcus erythropolis* IGTS8⁴¹. However, since *P. aeruginosa* does not utilize DBT, or other aromatic or aliphatic sulfides, as a sulfur source, M_{su}C must have a different role.

3.1.1. Taurine Degradation by *Pseudomonas* Species

Naturally occurring aliphatic sulfonates include taurine (2-aminoethanesulfonate), isethionate (2-hydroxyethanesulfonate), cysteate, methanesulfonate and sulfoquinovose. Taurine utilization as a sulfur source involves the α -ketoglutarate-dependent dioxygenase TauD. This enzyme has been best characterized from *E. coli*^{29, 32}, where it is an 81 kDa homodimer, with a K_m of 55 μ M for taurine. It is encoded together with an ABC-transporter for taurine (*tauABC*), and this configuration is conserved in several *Pseudomonas* species, including *P. aeruginosa*, *P. putida*, and *P. syringae* (*P. syringae* appears to contain two taurine transporters, only one of which is associated with a TauD homologue). The *P. aeruginosa* and *P. putida* TauD proteins are about 60% identical to that of *E. coli*, and α -ketoglutarate-dependent taurine desulfonation activity has been reported in *P. putida*¹³⁰, so it seems likely that the mechanism is similar to that in *E. coli*.

Although *P. putida* expresses a TauD-like activity, it does not rely on it for growth with taurine, since taurine is also accepted as substrate by the *P. putida* S_{su}D system¹²². This contrasts with *E. coli* S_{su}D, which does not display taurine desulfonation activity at all³⁰, a characteristic shared with *P. aeruginosa* M_{su}D⁶². Under sulfate starvation conditions, *P. putida* synthesizes a further α -ketoglutarate dependent dioxygenase, AtsK, which catalyzes the cleavage of sulfate esters (see below), but does not accept taurine as a substrate⁵³. Of the other natural sulfonates, cysteate is used as a sulfur source by *P. putida* S-313⁵⁵, but not by *P. putida* KT2440 or *P. aeruginosa* (Table 1), whereas isethionate could be desulfurized by all strains tested. Cysteate is not a substrate for the S_{su}D enzyme^{30, 122}, and an *ssu* mutant of *P. putida* S-313 was still able to grow with cysteate⁵⁵, so there is clearly an additional, uncharacterized sulfonatase present in this strain. By contrast, mutation of the *ssu* region of *P. putida* S-313 led to loss of the ability to grow with isethionate⁵⁵.

Taurine can also be used as a carbon source by some *P. aeruginosa* isolates¹⁰⁶, though not by *P. aeruginosa* PAO1¹⁰³. In *P. aeruginosa* TAU-5, taurine is transaminated to sulfoacetaldehyde, and the latter compound is then hydrolyzed to sulfite and acetate by a thiamine-pyrophosphate dependent sulfoacetaldehyde lyase¹⁰⁷. Expression of the lyase is co-induced with that of

taurine aminotransferase during growth with taurine. Sulfoacetaldehyde is a central intermediate in the metabolism not only of taurine, but also of isethionate, sulfoacetate and ethanedisulfonate^{25, 64, 65}.

3.2. Aromatic Sulfonates

Like aliphatic sulfonates, aromatic sulfonates can be used by bacteria as a source of carbon and energy or as a source of sulfur. *Pseudomonas* strains that can desulfurize arylsulfonates can be readily isolated from soils, and the pathways concerned have been best elucidated in *P. putida* S-313, a sewage isolate obtained by enrichment for growth with naphthalenesulfonate as sulfur source¹³⁸. This strain desulfurizes a wide range of arylsulfonates^{60, 99, 138} to yield the corresponding phenols and naphthols quantitatively—no degradation of the carbon skeleton is observed. Incorporation of ¹⁸O₂ into the product phenol indicated that the reaction is catalyzed by a monooxygenase¹³⁸, and mutant studies later revealed that the monooxygenase concerned is the same enzyme that cleaves aliphatic sulfonates, SsuD⁵⁵. Just as for alkanesulfonate cleavage, the reaction requires reduced FMN supplied by the SsuE protein, and sulfur is released as sulfite¹³¹.

Many organisms contain the *ssuD* gene, but most strains are not able to desulfurize aromatic sulfonates. *P. putida* S-313 contains an additional locus that allows the strain to use arylsulfonates (*asfRABC*). This locus encodes a LysR-type transcriptional regulator, AsfR, and three further proteins, AsfABC. AsfA is a 58.2-kDa protein that shows sequence homology to the flavoprotein subunit of fumarate reductases, and it is translationally coupled to AsfB, a 12-kDa ferredoxin containing two [4Fe-4S] clusters that are most closely related to ferredoxins known in anaerobic organisms. The role of these proteins is not yet clear, since the arylsulfonatase complex has not yet been reconstituted for biochemical characterization, but it is thought that they may accept reducing equivalents from FMNH₂, and transfer them to the oxygenase SsuD (Figure 3B). The third gene in the operon, *asfC*, encodes a periplasmic sulfonate-binding protein¹³¹ that is involved in the uptake of arylsulfonates into the cell. The membrane component of this transporter is AtsB, and the sulfate ester binding protein AtsR can replace AsfC to catalyze arylsulfonate transport¹³¹. Transformation of *P. aeruginosa* with the *asfAB* genes allowed growth with arylsulfonates as a sulfur source, but in an *atsR* mutant, growth only occurred when *asfC* was also supplied.

Interestingly, although *P. aeruginosa* PAO1 and *P. putida* KT2440 cannot desulfurize arylsulfonates, both contain an *asf*-like locus consisting of a fumarate reductase homologue, a ferredoxin and a sulfonate-binding protein, though the levels of similarity to AsfABC are quite low. Expression of the *asfC* homologue in *P. aeruginosa* (gene PA2296) was strongly upregulated during

sulfate starvation, and the protein was identified as a sulfate starvation-induced protein in a proteomic screen⁹¹. It seems possible, therefore, that in *P. putida* S-313, an existing, sulfur-regulated locus of unknown specificity has evolved to its current function as part of an arylsulfonate complex. Analysis of the regions flanking the *P. putida* S-313 *asfRABC* gene cluster indicates that these genes have been inserted into gene PP180 of the *P. putida* KT2440 genome sequence. This is close to, but not identical with, the *asfABC* homologues in strain KT2440 (genes PP205-PP207), suggesting that gene duplication and functional divergence may have occurred in strain S-313.

3.3. Utilization of Aromatic Sulfur Compounds as Carbon Sources

Arylsulfonates are used by a number of bacterial species as a source of carbon and energy for growth, and this has been reviewed in some detail in recent years^{20, 59}. The carbon-sulfur bond is usually cleaved early in the degradation pathway by a multicomponent dioxygenase system to release sulfite and the corresponding catechol. Enzymes of this nature that cleave toluenesulfonate and *p*-sulfobenzoate have been characterized in *Alcaligenes* sp. O-1⁵² and *Comamonas testosteroni* T-2⁷², respectively, and a broad substrate range dioxygenase that cleaves naphthalenesulfonates is known in *Sphingomonas xenophaga* BN6¹¹⁰. These enzymes are inducible, and the *C. testosteroni* genes encoding the sulfobenzoate dioxygenase (*psbAC*) are encoded on a putative transposon, flanked by two *IS1071* elements²⁰, probably located on the large plasmid pT2L¹²⁰. An enrichment study to isolate toluene-sulfonate-degrading strains from around the world identified a range of isolates, including *Pseudomonas* sp., and it is entirely plausible that the same desulfonation genes are also active in *Pseudomonas* strains. Other *Pseudomonas* strains that degrade benzene- or naphthalenesulfonates have been reported^{13, 70, 84-86}, but they have not all been systematically characterized and their taxonomic identity is not certain¹¹⁰.

Aromatic sulfonates are of special interest as dyestuffs and as surfactants. Several strains formerly designated as *Pseudomonas* have been isolated that can decolorize sulfonated azo dyes, but this is usually by reductive cleavage of the azo bond, and not by desulfonation¹⁰⁹. Consortia that are able to mineralize sulfonated azo dyes have been reported⁴⁴, but no individual *Pseudomonas* strains are known with this trait¹⁰⁹. This contrasts with the facile desulfonation of the dyestuff Orange II by *P. putida* S-313 when the compound is supplied as a sulfur source⁶⁰. Consortia rather than individual pseudomonads also seem to be required for the mineralization of sulfonated surfactants^{101, 102}, though one isolate of *P. aeruginosa*, strain W51D, can assimilate branched

chain alkylbenzenesulfonates¹⁵. The initial step in the degradation pathway in this species was desulfonation of the aromatic ring by a monooxygenase which has not yet been characterized. This is the identical reaction observed for sulfur-limited degradation of linear alkylbenzenesulfonates⁶⁰, and it will be interesting to see whether the two enzymes are related.

Benzothiophenes and DBTs make up a large proportion of the sulfur present in crude oil, and there is great interest in biological processes that can be used for desulfurization^{77, 79}. Several *Pseudomonas* strains have been isolated that can partially degrade DBT, by oxidation of one aromatic ring to a formylbenzothiophene derivative⁶⁸. These include *P. putida* RE204, which carries out a variety of transformations of benzothiophene²⁸, and several *Pseudomonas* isolates that have not been further characterized taxonomically. None of these isolates grow with condensed thiophenes as carbon source, and the transformations take place cometabolically⁶⁸. By contrast, desulfurization of DBT occurs by oxidation to DBT-dioxide, and a two-step cleavage to yield hydroxybiphenyl. This pathway is well known in a number of *Rhodococcus* species, where it is catalyzed by the products of the *dsz* genes, but it is not known in *Pseudomonas*. However, engineered *P. aeruginosa* and *P. putida* strains containing the *dsz* genes quantitatively desulfurized DBT, and grew rapidly with DBT as their sole sulfur source⁴⁰. Due to their solvent-tolerant nature, they may prove useful in biodesulfurization applications in the future.

4. SULFATE ESTERS AND SULFATASES

Like sulfonates, sulfate esters make up a great part of the sulfur present in soils. Much of this sulfate ester content undoubtedly arises through non-biological sulfation of lignin-derived phenols, but since many other biological molecules are sulfated (glycosaminoglycans, mucins, many mammalian excretion products) these also provide a source pool. Bacterial alkylsulfatases are involved in initial desulfation of sulfate esters to provide both sulfur and carbon for growth—the role of arylsulfatases, by contrast, is largely limited to the sulfur cycle²⁷. As can be seen in Table 1, of the sequenced laboratory strains of *Pseudomonas*, only *P. aeruginosa* PAO1 is able to grow with several different sulfate esters, although sulfatases of varying specificity have been characterized from several *Pseudomonas* species (Table 2). All the strains tested could grow with nitrophenylsulfate, which is the substrate usually used to test for soil arylsulfatase activity (used as a common marker of soil health), but the identity of the enzyme responsible is unclear. Indeed, the term “aryl-sulfatase” is itself misleading, since the natural substrates of human arylsulfatases are mainly carbohydrate sulfates and not aromatic sulfates¹³², and the same may well be true for bacterial arylsulfatases.

Table 2. Sulfatases and sulfotransferases in pseudomonads.

Organism	Enzyme	Gene	Substrate range	References
<i>Pseudomonas</i> sp. C ₁₂ B	P1 alkylsulfatase	— ^a	C ₆ –C ₁₂ alkylsulfate esters, nitrophenylsulfate	[6]
<i>Pseudomonas</i> sp. C ₁₂ B	P2 alkylsulfatase	—	C ₄ –C ₁₄ alkylsulfate esters	[19]
<i>Pseudomonas</i> sp. C ₁₂ B	S1, S2 and S3 secondary alkylsulfatases	—	S1—C ₈ –C ₁₀ (D)-sec. sulfate esters S2—C ₈ –C ₉ (L)-sec. sulfate esters S3—symmetrical sec. sulfate esters	[27], [76], [105]
<i>Pseudomonas</i> sp. C ₁₂ B	Arylsulfatase	—		[36]
<i>P. aeruginosa</i>	Alkylsulfatase	—		[38]
<i>P. putida</i> S-313	Alkylsulfatase	<i>atsK</i>	C ₄ –C ₁₂ alkylsulfate esters	[53]
<i>P. putida</i> S-313	Arylsulfotransferase	<i>astA</i>	Arylsulfate esters	[54]
<i>P. aeruginosa</i>	Arylsulfatase	<i>atsA</i>	Arylsulfate esters	[9], [24]
<i>P. aeruginosa</i>	Cholinesulfatase	<i>betC?</i>	Choline sulfate	[73]
<i>Pseudomonas</i> sp. ATCC 19151	SDS sulfatase	<i>sdsA</i>	SDS	[21]

^aGene not yet identified.

4.1. Alkylsulfatases

Aliphatic sulfate esters have been in use as surfactants for many years, and this has led to considerable research into their biodegradability. It is relatively easy to isolate strains from the environment that are able to use sulfate esters such as sodium dodecyl sulfate (SDS) as a carbon and energy source, both from contaminated and from pristine environments¹³⁵. The initial desulfation step has been best studied in *Pseudomonas* sp. C₁₂B, an SDS-utilizer that was originally isolated from soil adjacent to a sewage outlet⁸⁹. This strain contains a range of sulfatases which differ in their substrate specificity and their mode of regulation. It expresses two primary alkylsulfatases, designated P1 and P2^{6, 19}; P1 is a 181 kDa tetrameric protein that desulfates C₆–C₁₂ substrates, with K_m values decreasing with chain length, from 4.7 mM (C₆) to 68 μ M (C₁₂). It has optimum activity at pH 6.1, and interestingly, it is also able to cleave nitrophenylsulfate⁶. The P2 enzyme is a 160 kDa dimer, and has a higher pH optimum (pH 8.3). The range of substrates accepted is similar to P1, as are the K_m values (12.5 mM [C₆] to 30 μ M [C₁₂]), though it does not react with arylsulfates¹⁹. Three further alkyl sulfatases have also been identified in strain C₁₂B. These are specific for secondary alkylsulfates, which are not substrates for P1 and P2, and catalyse the cleavage of D-, L- and symmetrical

alkyl-2-sulfates, respectively. There is very little genetic information available about these enzymes, and so it is difficult to assess how widely they are distributed in this and other genera.

Enrichment for utilization of SDS has also yielded other strains, including *Pseudomonas* sp. ATCC 19151⁴⁵, for which more is known at a genetic level. Complementation of a transposon mutant of this strain that was no longer able to use SDS as a carbon source for growth led to the identification of *sdsA* as the gene encoding the SDS sulfatase²¹. *sdsA* encodes a protein of the lactamase family, and is located divergently to a LysR-family regulator gene (*sdsB*) that is also required for growth with SDS. Overproduction of the homologous *P. aeruginosa* SdsA protein (gene *PA0740*) in *E. coli* gave an active sulfatase with an N-terminal sequence that was very similar to that determined for the *Pseudomonas* sp. C₁₂B P2 sulfatase (unpublished). It therefore appears that *sdsA* corresponds to the P2 enzyme of *Pseudomonas* sp. C₁₂B.

The alkylsulfatases described above are specific for medium-long chain sulfate esters, and for utilization of the ester as a carbon source—indeed, batch cultures of *Pseudomonas* sp. C₁₂B do not synthesize the P1 enzyme when grown with citrate as carbon source and hexylsulfate as sulfur source³⁵. Other sulfatases must therefore be induced to supply sulfate for growth under these conditions. One such enzyme has been identified in *P. putida* S-313. This enzyme is the product of the *atsK* gene, and is a 121 kDa tetrameric α -ketoglutarate-dependent dioxygenase that is quite closely related to TauD (39% identity to *P. putida* TauD). However, AtsK does not cleave taurine, but instead releases sulfate from a range of aliphatic sulfate esters (C₄–C₁₂), with K_m values between 34 μ M and 400 μ M. The reaction requires oxygen and Fe²⁺, and generates the corresponding aldehyde as a product⁵³. Interestingly, the specific activity of the enzyme reported was relatively low in comparison to that of *E. coli* TauD, which reflects the fact that the enzyme carries out oxygenolytic cleavage at the relatively unactivated carbon atom adjacent to a sulfate ester group, compared to the activated position adjacent to the sulfonate moiety in taurine. The *atsK* gene is also present in *P. aeruginosa* (apparently in two copies adjacent to each other, though it is not known if both are active), and its expression was found to be strongly upregulated in this species during growth under sulfate-limiting conditions⁹¹. The *ats-sft* cluster of *P. putida* S-313 (Figure 5) is not present in *P. putida* KT2440, but is located in the genome upstream of the *gabD* gene.

From sequence analysis, AtsK is a cytoplasmic protein, whereas SdsA is periplasmic, as is the P2 sulfatase from *Pseudomonas* sp. C₁₂B¹¹⁷. In *P. aeruginosa*, mutants in the *atsRBC* ABC-type sulfate ester transporter associated with the arylsulfatase were studied. Insertion mutants in both *atsR* (the putative periplasmic substrate binding protein) and *atsB* (the permease

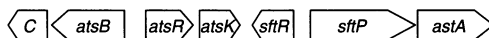
P. aeruginosa* PAO1**P. putida* S-313**

Figure 5. Sulfatase loci in *Pseudomonas* species. The sulfatase clusters in *P. aeruginosa* (PA0181-PA0194) and *P. putida* S-313 (acc. no. AF126201) are broadly similar, showing a clustering of genes which are involved in desulfurization of aryl and alkylsulfate esters. *P. putida* KT2440 and *P. syringae* lack these gene clusters.

component) of *P. aeruginosa* were no longer able to grow with hexylsulfate, but retained the ability to use SDS as a sulfur source⁴⁷. Identical results were obtained with *atsR* and *atsB* mutants of *P. putida* S-313⁵⁴. This is consistent with a model in which desulfurization of medium chain-length sulfate esters in *P. aeruginosa* is dependent on *AtsK* within the cell and the presence of the *AtsRBC* transport system, whereas SDS cleavage is catalyzed by the periplasmic *SdsA* protein. In *P. putida*, by contrast, *AtsK* is also required for SDS utilization⁵³, suggesting that a functional *sdsA* homologue is not expressed under these conditions in this species (the genome sequence reveals a putative *sdsA* homologue [32% identity to *P. aeruginosa sdsA*], but possibly this is not correctly expressed under sulfate starvation conditions). SDS transport may be catalyzed either by *AtsRBC*, or may occur by an unspecific mechanism.

Very little is known about the transport of sulfonates and sulfate esters across the outer cell membrane. Porin genes are present in the *P. aeruginosa* *ats* cluster (*opdI*), and immediately upstream of the *lsfA/ssu* cluster in *P. putida* (an *OprE* homologue) and *P. syringae* (an *OprD* homologue). It is noteworthy that the *P. putida* S-313 *ats/sft* gene cluster includes a gene for a putative TonB-dependent receptor protein (*SftP*) whose synthesis is upregulated under sulfate-limited growth conditions⁵⁴. This gene is also present in *P. aeruginosa*, and is closely associated with the *tonB2* gene and genes for TonB accessory proteins *ExbB* and *ExbD*¹³⁷. These may be involved in the import of sulfur-containing substrates, but this has not yet been investigated in detail.

4.2. Arylsulfatases

Like aliphatic sulfate esters, arylsulfates also require an active transport system to allow them to enter the cell. In *P. aeruginosa* and *P. putida* S-313, this transport system is the same ABC-type transporter that is responsible for transport of alkyl sulfate esters and arylsulfonates, *AtsRBC*. *AtsR* constitutes

the periplasmic substrate-binding protein, which interacts with the core permease of the transporter, AtsB. At 57.7 kDa, AtsB is somewhat larger than many other permease proteins of ABC-type transporters, and it appears to be made up of twelve transmembrane helices rather than the more usual six. It seems likely, therefore, that a monomer of AtsB interacts with two AtsC proteins (the ATP-binding subunit of the complex) to form the active transporter.

Cleavage of aromatic sulfates is catalyzed by arylsulfatases, yielding the corresponding phenol and sulfate. Early work by Harada⁴³ identified at least three sulfatase enzymes in *P. aeruginosa*, including a choline sulfatase, and further, more detailed research led to the characterization of two arylsulfatase isozymes, with slightly different pH optima^{23, 24}. Biochemical studies later indicated that the main arylsulfatase in *P. aeruginosa* PAO1 is the product of the *atsA* gene⁹. This 60 kDa monomeric enzyme accepts a number of arylsulfates as substrate (K_m 105 μ M for nitrocatecholsulfate), but does not desulfurize alkylsulfates, and has an activity pH optimum at 8.9. This contrasts with the other well-characterized bacterial arylsulfatase, from *Klebsiella pneumoniae*, which has optimal activity at pH 7.1 (see ref. [94]). More importantly, however, the arylsulfatase of *K. pneumoniae* is a periplasmic enzyme^{78, 80}, whereas that of *P. aeruginosa* is intracellular. This seems surprising, since arylsulfatases are generally thought to be involved in scavenging sulfate-ester sulfur under low sulfate conditions²⁷, but is confirmed by the need for an intact AtsRBC sulfate ester transporter for growth of *P. aeruginosa* with arylsulfates⁴⁷.

Like other characterized arylsulfatases, *P. aeruginosa* AtsA contains a conserved sequence motif in the active site, which is required for enzyme activity. This sulfatase motif (C/S-X-P-X-R-X₄-T-G) directs the post-translational oxidation of the initial cysteine or serine in the sequence into an α -formylglycine residue. The presence of a cysteine in the sulfatase motif is associated in all bacterial sulfatases with the absence of a signal peptide, and the *cys*-type arylsulfatases are therefore thought to be intracellular. Conversely, enteric bacteria, in particular, possess arylsulfatases with a serine in the preprotein, and this characteristic is associated with sulfatases that are located in the periplasm. The mechanism by which cysteine is converted to formylglycine is not yet known. An iron-sulfur protein (AtsB—not related to the *P. aeruginosa* AtsB that is a component of the sulfate ester transporter) that is essential for the Ser-fGly conversion has been identified in *K. pneumoniae*¹¹³. However, this protein was not involved in the maturation of *P. aeruginosa* *cys*-type arylsulfatase. A mutant *P. aeruginosa* sulfatase in which the active cysteine was replaced by a serine (C51S-AtsA²⁶) was not active in either *P. aeruginosa* or *E. coli*, although the *K. pneumoniae* *ser*-type sulfatase was correctly modified. Activation of the *cys*- and *ser*-type sulfatases therefore seem to require separate systems. There are no known *ser*-type sulfatases in *Pseudomonas*, and *P. aeruginosa* does not contain a homolog of *K. pneumoniae* AtsB.

Structural analysis of the *P. aeruginosa* AtsA protein has shed light on the role of the formylglycine in the sulfatase mechanism¹¹. In the resting state of the enzyme, this residue is present as its hydrate (determined at 1.3 Å), and the active site is very similar to that seen in the human arylsulfatase A and B^{12, 74}. Cleavage of sulfate esters occurs by nucleophilic attack of the fGly hydrate on the sulfur atom of the ester, releasing the alcohol product. This mechanism constitutes cleavage of the S–O bond of the sulfate ester, which contrasts with the unusual C–O cleavage that has been observed with alkylsulfatases such as the P2 enzyme of *Pseudomonas* sp. C₁₂B, and secondary alkylsulfatases in the same strain⁶. Interestingly, the P1 alkylsulfatase in this strain also cleaves the S–O bond during sulfate ester hydrolysis⁶, and it may well be more closely related to arylsulfatases than to alkylsulfatases of the SdsA type.

Analysis of genomic sequence data from *Pseudomonas* species shows that *P. aeruginosa* contains genes for two further *cys*-type sulfatases in addition to AtsA. One of these is very similar to known choline sulfatases, and probably represents the choline sulfatase reported in this species⁷³. The other additional sulfatase has not yet been characterized, but may represent the second arylsulfatase isozyme previously described²⁴. However, since an *atsA* mutant of *P. aeruginosa* PAO1 was unable to desulfurize arylsulfates²⁶, the second isozyme may be cryptic in this strain. *P. putida* KT2440 is unable to grow with arylsulfates other than nitrophenylsulfate as sulfur source (Table 1), and it is therefore not unexpected to find that it is lacking the *atsRBC* transporter. A gene encoding a putative *cys*-type sulfatase is present in the genome (43% identical to *P. aeruginosa* AtsA), but unusually this seems to carry a signal peptide, and it is not clear whether it is correctly expressed and processed. Complementation of strain KT2440 with the *P. aeruginosa* PAO1 sulfate ester transporter (*AtsRBC*) did not allow growth with nitrocatecholsulfate, suggesting that the enzyme is not active in this species (unpublished). Arylsulfatases have not yet been studied in detail in other pseudomonads, but the genomic data available indicate that *P. fluorescens* has an arylsulfatase gene very similar to that of *P. aeruginosa*, also associated with a sulfate ester transporter, and two other *cys*-type sulfatases. One *cys*-type sulfatase gene is found in *P. syringae*, which is probably a choline sulfatase.

As mentioned above, *P. putida* KT2440 is unable to grow with aromatic sulfate esters as a sulfur source, but the related strain *P. putida* S-313 can do so efficiently. Screening of transposon mutants that had lost the ability to desulfurize aromatic sulfates showed that they had insertions either in the sulfate ester transporter or in a putative arylsulfotransferase, encoded by the *astA* gene⁵⁴. The AstA protein is similar (43–44% identity) to periplasmic sulfotransferases that have been characterized in enteric bacteria^{4, 56}. It catalyses the transfer of sulfate from a sulfate ester (e.g., nitrocatecholsulfate) to a phenol acceptor, and shows highest activity with dihydroxy acceptors such as catechol

or dopamine⁵⁵. In related sulfotransferases from enteric bacteria, sulfuryl transfer is achieved via a sulfoenzyme intermediate¹⁶, with the sulfo group transferred to an active site tyrosine⁶⁹. This tyrosine is conserved in *P. putida* AstA (Tyr-112), and presumably forms the active centre, though tyrosine and tyrosine-containing peptides did not make good acceptor substrates for the enzyme⁵⁵. The natural substrates of this enzyme have not yet been identified, but presumably sulfate is transferred onto an acceptor that can be readily hydrolysed (either spontaneously or using a specific sulfatase) to provide sulfate for cysteine biosynthesis.

4.3. Choline and Bile Acid Sulfatases

A choline sulfatase activity was identified in *Pseudomonas* sp. in early reports^{37, 43}, and the *P. aeruginosa* enzyme has been characterized biochemically⁷³. It has a K_m of 1.4 mM towards choline sulfate, and is differentially expressed depending on whether the carbon, nitrogen or sulfur of the substrate is being utilized. Although no sequence data has been obtained, it is almost certainly encoded by the *betC* gene, homologous to that of *Sinorhizobium meliloti*⁸⁷. All four sequenced *Pseudomonas* genomes contain homologues to *S. meliloti* BetC (45–48% identical). In *S. meliloti*, BetC is required for the synthesis of glycine betaine, which is required by the cell as an osmoprotectant¹⁰⁸. Since *P. aeruginosa* also uses glycine betaine as an osmoprotectant⁷¹, choline sulfatase is probably also a part of the glycine betaine synthetic pathway in this species. Although choline sulfatase is in principle an alkylsulfatase, it contains a *cys*-type formylglycine modification motif, and is closely related at sequence level to the arylsulfatase family.

A *P. aeruginosa* isolate has been shown to utilize lithocholic acid sulfate as a sulfur source⁴⁸, though the sulfatase responsible was not identified. However, bile acid sulfatases have been identified in *C. testosteroni* ATCC 11996 (acc. no. AAB44526; refs [114], [116]), and are related to SdsA. The lithocholic acid sulfatase was a homodimer of 53 kDa subunits, with a pH optimum at 8.5. Unlike other characterized sulfatases, this enzyme was Mn^{2+} -dependent, and contained 1–2 atoms of manganese per protein molecule¹¹⁵.

4.4. Carbohydrate and Mucin Sulfatases

In cystic fibrosis patients, the level of sulfation of respiratory mucin is greatly increased, leading to increased acidity and resulting changes in the physical properties of the mucus¹¹². The sulfate in sulfomucin is found on galactose residues (both internal and terminal), *N*-acetylglucosamine and occasionally *N*-acetylgalactosamine residues^{17, 51}. Mucin sulfation is known to prevent bacterial degradation of the mucin by glycosidases and proteinases¹²³. *P. aeruginosa* isolates grew poorly with a range of sulfated sugars as carbon

source, compared with the unsulfated forms, suggesting that sulfation may play an important role in preventing bacterial colonization¹⁷.

However, *P. aeruginosa* also displays a chemotactic response to components of human respiratory mucin⁸¹, and has been shown to bind to the di- and trisaccharide structures that are part of mucin side chains. This may play an important role in colonization⁹⁵. A necessary step in initiating this adhesion process is probably desulfation, to allow partial deglycosylation of the mucin and uncover potential binding epitopes⁵¹. A mucin sulfatase activity has been reported in *P. aeruginosa* and also in a number of *Burkholderia cepacia* strains⁵¹. This activity seems to be independent of the characterized arylsulfatase activity in these strains, suggesting that a novel mechanism for desulfation may be involved. Mucin sulfatase activity was highest in a cystic fibrosis isolate, *P. aeruginosa* E601, and no activity was found in *P. aeruginosa* PAO1. The enzyme responsible for the activity has not yet been identified, though the recent development of a mucin sulfatase assay¹⁸ may aid this process. The only mucin sulfatase identified to date is from *Prevotella* sp. RS2¹³⁶, which is a *ser*-type arylsulfatase with low homology to known *Pseudomonas* arylsulfatases.

4.5. Sulfate Esters and Soil Bacteria

Sulfate esters are common not only in human respiratory mucin, but are also an important component of the sulfur cycle in other environments. Aerobic soils contain less than 5% of their sulfur content as inorganic sulfate³, and sulfate esters make up over 50% of the sulfur pool¹³³. Microbial sulfatases in the soil catalyse the hydrolysis of these sulfate esters, and as a result, soil sulfate-ester sulfur is almost as accessible to plants as adsorbed inorganic sulfate¹⁰⁴. The ability to desulfurize sulfate esters is crucial for bacterial growth in the soil as well. A mutant of *P. putida* S-313 containing a mutated *sfiR* gene, which encodes a LysR-family regulator that controls expression of a number of sulfatases in this strain, survived significantly less well in a range of soils compared to the wildtype strain⁵⁴. Utilization of the soil sulfate esters is therefore important, but more work is needed to determine which sulfatase genes are expressed during growth in soils and rhizosphere.

5. REGULATION OF ORGANOSULFUR METABOLISM

5.1. CysB and Global Sulfur Regulation

The sulfate assimilation pathway has been characterized in detail in enteric bacteria⁶⁷, and the biochemistry is broadly similar in *Pseudomonas* spp.,

though there are some differences, as detailed above. Expression of the genes of the cysteine biosynthetic pathway is strongly upregulated in enteric bacteria under sulfate-limited conditions, and this activation is modulated by the CysB transcriptional activator. When the *P. aeruginosa* *cysB* gene was mutated, the resulting strain was auxotrophic for cysteine²², confirming that this protein is also required for cysteine biosynthesis in this species. However, the *cysB* mutant could still grow with sulfide or sulfite as the sulfur source⁶², and was also able to utilize methionine or homocysteine by the reverse transsulfurylation pathway. Reduction of sulfite to sulfide therefore appears to be not completely controlled by CysB—since a *cysI* mutant is auxotrophic for cysteine⁴⁶, this suggests that sulfite reductase expression is differently controlled to enteric bacteria. Likewise, reverse transsulfurylation is CysB-independent.

CysB is also crucially involved in uptake and desulfurization of sulfonates and sulfate esters. In *P. aeruginosa*, expression of both the *atsR* and *atsBCA* transcriptional units of the sulfate ester transporter requires CysB⁴⁷. CysB was also involved in the expression of *sfnEDR* operon in *P. putida* DS1³³, and was required for the growth of *P. aeruginosa* with methanesulfonate⁶². As well as alkanesulfonates and sulfate esters, a *P. aeruginosa* *cysB* mutant was unable to desulfurize cyclamate, though the pathway catalyzing this process is not yet known.

In *P. aeruginosa*, CysB is not only involved in the regulation of cysteine biosynthesis, but also plays a part in alginate production. The CysB protein binds to a specific sequence in the promoter of the *algD* gene²², which encodes GDP-mannose dehydrogenase, the key enzyme in alginate synthesis. Inactivation of the *cysB* gene led to a reduction in *algD* expression, though no apparent effect on mucoidy was observed in the mutant strain. It is not yet clear why CysB, which is primarily known as a transcriptional activator of the *cys* genes, is active in alginate synthesis.

CysB is a member of the LysR-family of transcriptional activators, and in *E. coli* it acts as a homotetramer of 36 kDa subunits, binding to the target DNA via a helix-turn-helix motif in the N-terminal domain. Activation of the *cys* genes requires *N*-acetylserine as co-activator, while sulfide and thiosulfate act as corepressors⁶⁷. The CysB protein of *P. aeruginosa* is quite similar to that of *E. coli* (64% identity), and homologous proteins have been found in all the sequenced pseudomonads (89–92% identity to *P. aeruginosa* CysB). *E. coli* also possesses a second, closely related regulator, the Cbl protein (for CysB-like), which is required for sulfonate metabolism^{127, 129}. As described below, the *cbl* gene has not been identified in *Pseudomonas* spp., but several other LysR-type regulators that are less closely related to CysB are indeed involved in organosulfur metabolism.

5.2. Other Regulators of Organosulfur Metabolism

Three other LysR-family regulators have been discovered in *Pseudomonas* that are known to be involved in organosulfur metabolism (Table 3). These are quite similar to each other, but do not show high levels of identity to CysB, and all show the expected helix-turn-helix DNA-binding domain in the N-terminal region. They differ in target specificity, but in at least one case there is evidence for cross-talk between them.

The first of these regulators, SftR, was identified in a mutant of *P. putida* S-313 that had lost the ability to grow with sulfate esters⁵⁴. Analysis of this mutant revealed that the SftR protein is required for expression of all the transcriptional units in the *ats/sft* cluster, including the *atsRBCA* sulfate ester transporter, the sulfatase *atsK*, and *sftP-astA*, encoding a putative TonB-dependent receptor and the arylsulfotransferase. *sftR* expression was repressed in the presence of inorganic sulfate, suggesting that it might itself be under the control of CysB, a feature that is also seen for the *cbl* gene in *E. coli*¹²⁷. A homologue of *sftR* is present in *P. aeruginosa* (gene PA191), and a mutant in which this gene was inactivated was unable to grow with aliphatic sulfate esters or with cyclamate, though it could still grow with aromatic sulfates⁵⁴. Sequence analysis reveals the presence of a second *sftR* orthologue in *P. aeruginosa* (PA2334; 54% identical to SftR), which is associated with an uncharacterized arylsulfatase gene, and it is possible that this regulator protein controls arylsulfatase expression in this species. *P. putida* KT2440 does not contain an SftR homologue, in accordance with its lack of sulfate ester utilization genes.

In a *P. putida* S-313 *sftR* mutant, no expression of arylsulfate ester uptake is observed, except when arylsulfonates are present. This is thought to be due to the presence of a further LysR-type regulator in this strain, AsfR,

Table 3. Regulators of sulfur metabolism in pseudomonads.

Regulator protein	Species	Target	References
CysB	<i>P. aeruginosa</i> and others	Sulfate assimilation	[22], [47]
SdsB	<i>P. aeruginosa</i> , <i>Pseudomonas</i> sp. ATCC 19151	SDS metabolism	[21]
AsfR	<i>P. putida</i> S-313	Aryldesulfonation	[131]
SftR	<i>P. putida</i> S-313, <i>P. aeruginosa</i>	Desulfurization of alkylsulfate esters and sulfamates	[54]
SfnR	<i>P. putida</i> DS1	Dimethylsulfone metabolism	[33]

which controls expression of the *asfABC* operon. Expression of *asfABC* is strongly upregulated in the presence of sulfonate substrates, and repressed by sulfate in a CysB-mediated manner. Rather unexpectedly for a LysR-type regulator, AsfR acts by repressing *asfA* unless a substrate is present, in which case repression is relieved. This unusual mode of action may be due to the fact that AsfA appears to be toxic to the cell unless its synthesis is carefully controlled. The dual regulation of *asfA* by CysB and AsfR again highlights a common theme, of very tightly regulated target operons in organosulfur metabolism. This is also seen for the *tau* and *ssu* operons in *E. coli*, which are controlled by both CysB and Cbl for expression¹²⁷—it is not yet known how *ssu* is regulated in *Pseudomonas*.

A further LysR-type regulator that is involved in organosulfur metabolism in *Pseudomonas* is SdsB, which is required for desulfurization of SDS by *Pseudomonas* sp. ATCC 19151 (see ref. [21]). It is located divergently to the *sdsA* gene, and presumably controls *sdsA* expression. *P. aeruginosa* contains a homologue of SdsB (64% identity), but a mutant in this gene grew normally with SDS as the sulfur source (unpublished). This is probably due to AtsK-mediated SDS desulfurization in this species, but this remains to be confirmed.

All the transcriptional regulators described above belong to the LysR superfamily, and these seem to dominate the regulation of organosulfur metabolism. However, *P. putida* DS1 requires an NtrC-type activator (SfnR) for growth with DMS. Transcriptional activators of this type use σ^{54} -RNA polymerase in transcription, and inactivation of the *rpoN* gene indeed abolished the ability of the strain to convert dimethylsulfone to methanesulfonate³³. Deletion of the *sfnR* gene did not, however, affect transcription of *sfnECR*, and so although sequence analysis reveals a conserved putative RpoN-dependent promoter sequence upstream of *sfnE*, it seems not to be controlled by SfnR. This promoter sequence motif is also conserved in the *P. putida* KT2440 and *P. aeruginosa* *msuEDC* clusters. In *P. aeruginosa*, there is an additional SfnR homologue (64% identical to *P. putida* SfnR) encoded immediately upstream of *msuE*, and it will be interesting to evaluate the role this plays in sulfonate metabolism.

LysR-type regulators are involved in controlling organosulfur metabolism even when the sulfur-containing compound is being used as a carbon source, rather than purely to provide sulfur for growth. The SdsB protein described above is required for growth of *Pseudomonas* sp. ATCC 19151 (see ref. [21]) with SDS as the carbon source. In *C. testosteroni* T-2, the key regulator controlling mineralization of toluenesulfonate is TsaR, a LysR-type regulator encoded divergently to the genes for the multicomponent oxygenase system that catalyses toluenesulfonate cleavage, *tsaMBCD*¹²¹. TsaR interacts with toluenesulfonate to bind to three specific regions in the *tsaM* promoter region, and is reversibly inactivated by oxygen. The entire gene cluster is located on a transposon carried on the 72-kb plasmid pTSA. This plasmid, with its

conserved *tsa* genes, has been isolated from a number of toluenesulfonate-utilizing isolates from around the world, including several *Pseudomonas* strains¹²⁰.

5.3. How is Sulfur Limitation Sensed?

Many of the enzyme systems described above for the metabolism of sulfonates and sulfate esters were first identified as proteins whose synthesis was upregulated in the absence of sulfate, and repressed during growth with inorganic sulfate^{46, 91}. But how does the cell detect the presence of sulfate? In *E. coli*, sulfate is not itself a corepressor of *cys* gene induction. However, its reduction product, sulfide, acts as an anti-inducer, binding to CysB to displace the co-inducer *N*-acetylserine. In *P. aeruginosa*, the situation is more complex. Expression of *atsA* and *msuD* was studied in strains that were mutated in the *cysI* and *cysN* genes^{46, 62}. Expression of *atsA* was not repressed by sulfate in a *cysN* background, confirming that sulfate is also not a direct corepressor here. However, *atsA* was not expressed in a *cysI* background in the presence of either sulfite or sulfide, demonstrating that at least two intermediates of the *cys* pathway act as corepressors (APS/sulfite and cysteine/sulfide). Similar results were obtained for *msu* expression, with the exception that sulfate itself also acted directly as a corepressor of *msu* expression, even in a *cysN* mutant⁶².

In *E. coli*, it has been shown that the corepressor associated with the Cbl protein is not sulfide, as for CysB, but APS¹⁴. Regulation of the *ssu* operon has not yet been examined in detail in pseudomonads, but the above data obtained with *P. aeruginosa* *msu* and *ats* is consistent with a model where APS is also a key co-effector of organosulfur metabolism in pseudomonads.

6. CONCLUSIONS

Due to the variety of environments that they inhabit, *Pseudomonas* species show great metabolic flexibility, reflected in the high number of regulator proteins encoded in their genomes^{82, 111}. As shown above, this flexibility extends to their use of a wide variety of organic and inorganic sulfur compounds to provide sulfur for growth. The biochemical mechanisms by which this organically bound sulfur is assimilated are largely conserved between *Pseudomonas* species. However, examination of the gene loci concerned confirms the genetic plasticity of the genus, since although many of the genes are well conserved, their organization varies, even between strains of the same species (cf. Figure 4). Where it has been examined, expression of these genes has been found to be tightly regulated, both on a global level by the CysB protein and at the level of specific regulators that recognize the particular substrates concerned. However, although we have learnt a great deal about the metabolism of these

substrates in the last few years, much more remains to be discovered. Microarray analysis of *P. aeruginosa* grown under sulfate-limited conditions (unpublished) reveals that expression of several uncharacterized transport systems, and of a number of other proteins is strongly upregulated. This suggests that this species is also able to recognize and metabolize other groups of sulfur compounds, whose identification will provide further challenges for future studies.

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REFERENCES

1. Alaminos, M. and Ramos, J.L., 2001, The methionine biosynthetic pathway from homoserine in *Pseudomonas putida* involves the *metW*, *metX*, *metZ*, *metH* and *metE* gene products. *Arch. Microbiol.*, 176:151–154.
2. Andersen, G.L., Beattie, G.A., and Lindow, S.E., 1998, Molecular characterization and sequence of a methionine biosynthetic locus from *Pseudomonas syringae*. *J. Bacteriol.*, 180:4497–4507.
3. Autry, A.R. and Fitzgerald, J.W., 1990, Sulfonate S—A major form of forest soil organic sulfur. *Biol. Fertil. Soils*, 10:50–56.
4. Baek, M.C., Kim, S.K., Kim, D.H., Kim, B.K., and Choi, E.C., 1996, Cloning and sequencing of the *Klebsiella* K-36 *astA* gene, encoding an arylsulfate sulfotransferase. *Microbiol. Immunol.*, 40:531–537.
5. Baker, S.C., Kelly, D.P., and Murrell, J.C., 1991, Microbial degradation of methanesulfonic acid—a missing link in the biogeochemical sulfur cycle. *Nature*, 350:627–628.
6. Bateman, T.J., Dodgson, K.S., and White, G.F., 1986, Primary alkylsulfatase activities of the detergent-degrading bacterium *Pseudomonas* C₁₂B: Purification and properties of the P1 enzyme. *Biochem. J.*, 236:401–408.
7. Beattie, G.A. and Lindow, S.E., 1994, Comparison of the behavior of epiphytic fitness mutants of *Pseudomonas syringae* under controlled and field conditions. *Appl. Environ. Microbiol.*, 60:3799–3808.
8. Beattie, G.A. and Lindow, S.E., 1994, Survival, growth, and localization of epiphytic fitness mutants of *Pseudomonas syringae* on leaves. *Appl. Environ. Microbiol.*, 60:3790–3798.
9. Beil, S., Kehrli, H., James, P., Staudenmann, W., Cook, A.M., Leisinger, T., and Kertesz, M.A., 1995, Purification and characterization of the arylsulfatase synthesized by *Pseudomonas aeruginosa* PAO during growth in sulfate-free medium and cloning of the arylsulfatase gene (*atsA*). *Eur. J. Biochem.*, 229:385–394.
10. Bick, J.A., Dennis, J.J., Zylstra, G.J., Nowack, J., and Leustek, T., 2000, Identification of a new class of 5'-adenylylsulfate (APS) reductases from sulfate-assimilating bacteria. *J. Bacteriol.*, 182:135–142.

11. Boltes, I., Czapińska, H., Kahnert, A., von Bülow, R., Dierks, T., Schmidt, B., von Figura, K., Kertesz, M.A., and Usón, I., 2001, 1.3 Å crystal structure of arylsulfatase from *Pseudomonas aeruginosa* establishes the catalytic mechanism for sulfate ester cleavage in the sulfatase family. *Structure*, 9:483–491.
12. Bond, C.S., Clements, P.R., Ashby, S.J., Collyer, C.A., Harrop, S.J., Hopwood, J.J., and Guss, J.M., 1997, Structure of a human lysosomal sulfatase. *Structure*, 5:277–289.
13. Brilon, C., Beckmann, W., Hellwig, M., and Knackmuss, H.J., 1981, Enrichment and isolation of naphthalenesulfonic acid-utilizing pseudomonads. *Appl. Environ. Microbiol.*, 42:39–43.
14. Bykowski, T., van der Ploeg, J.R., Iwanicka-Nowicka, R., and Hryniewicz, M.M., 2002, The switch from inorganic to organic sulphur assimilation in *Escherichia coli*: Adenosine 5'-phosphosulphate (APS) as a signalling molecule for sulphate excess. *Mol. Microbiol.*, 43:1347–1358.
15. Campos-García, J., Esteve, A., Vázquez-Duhalt, R., Ramos, J.L., and Soberón-Chávez, G., 1999, The branched-chain dodecylbenzene sulfonate degradation pathway of *Pseudomonas aeruginosa* W51D involves a novel route for degradation of the surfactant lateral alkyl chain. *Appl. Environ. Microbiol.*, 65:3730–3734.
16. Chai, C.L.L. and Lowe, G., 1992, The mechanism and stereochemical course of sulfuryl transfer catalyzed by the aryl sulfotransferase from *Eubacterium* A-44. *Bioorg. Chem.*, 20:181–188.
17. Chance, D.L. and Mawhinney, T.P., 2000, Carbohydrate sulfation effects on growth of *Pseudomonas aeruginosa*. *Microbiology*, 146:1717–1725.
18. Clinch, K., Evans, G.B., Furneaux, R.H., Rendle, P.M., Rhodes, P.L., Robertson, A.M., Rosendale, D.I., Tyler, P.C., and Wright, D.P., 2002, Synthesis and utility of sulfated chromogenic carbohydrate model substrates for measuring activities of mucin-desulfating enzymes. *Carbohydr. Res.*, 337:1095–1111.
19. Cloves, J.M., Dodgson, K.S., White, G.F., and Fitzgerald, J., 1980, Purification and properties of the P-2 primary alkylsulfohydrolase of the detergent-degrading bacterium *Pseudomonas* C-12B. *Biochem. J.*, 185:23–32.
20. Cook, A.M., Laue, H., and Junker, F., 1999, Microbial desulfonation. *FEMS Microbiol. Rev.*, 22:399–419.
21. Davison, J., Brunel, F., Phanopoulos, A., Prozzi, D., and Terpstra, P., 1992, Cloning and sequencing of *Pseudomonas* genes determining sodium dodecyl sulfate biodegradation. *Gene*, 114:19–24.
22. Delic-Attree, I., Toussaint, B., Garin, J., and Vignais, P.M., 1997, Cloning, sequence and mutagenesis of the structural gene of *Pseudomonas aeruginosa* CysB, which can activate *algD* transcription. *Mol. Microbiol.*, 24:1275–1284.
23. Delisle, G. and Milazzo, F.H., 1970, The isolation of arylsulphatase isoenzymes from *Pseudomonas aeruginosa*. *Biochim. Biophys. Acta*, 212:505–508.
24. Delisle, G.J. and Milazzo, F.H., 1972, Characterization of arylsulfatase isoenzymes from *Pseudomonas aeruginosa*. *Can. J. Microbiol.*, 18:561–568.
25. Denger, K. and Cook, A.M., 2001, Ethanedisulfonate is degraded via sulfoacetaldehyde in *Ralstonia* sp. strain EDS1. *Arch. Microbiol.*, 176:89–95.
26. Dierks, T., Miech, C., Hummerjohann, J., Schmidt, B., Kertesz, M.A., and von Figura, K., 1998, Posttranslational formation of formylglycine in prokaryotic sulfatases by modification of either cysteine or serine. *J. Biol. Chem.*, 273:25560–25564.
27. Dodgson, K.S., White, G.F., and Fitzgerald, J.W., 1982, Sulfatases of microbial origin. CRC Press, Boca Raton.
28. Eaton, R.W. and Nitterauer, J.D., 1994, Biotransformation of benzothiophene by isopropylbenzene-degrading bacteria. *J. Bacteriol.*, 176:3992–4002.
29. Eichhorn, E., van der Ploeg, J.R., Kertesz, M.A., and Leisinger, T., 1997, Characterization of α -ketoglutarate dependent taurine dioxygenase from *Escherichia coli*. *J. Biol. Chem.*, 272:23031–23036.

30. Eichhorn, E., van der Ploeg, J.R., and Leisinger, T., 1999, Characterization of a two-component alkanesulfonate monooxygenase from *Escherichia coli*. *J. Biol. Chem.*, 274:26639–26646.
31. Eichhorn, E., van der Ploeg, J.R., and Leisinger, T., 2000, Deletion analysis of the *Escherichia coli* taurine and alkanesulfonate transport systems. *J. Bacteriol.*, 182:2687–2695.
32. Elkins, J.M., Ryle, M.J., Clifton, I.J., Hotopp, J.C.D., Lloyd, J.S., Burzlaff, N.I., Baldwin, J.E., Hausinger, R.P., and Roach, P.L., 2002, X-ray crystal structure of *Escherichia coli* taurine/alpha-ketoglutarate dioxygenase complexed to ferrous iron and substrates. *Biochemistry*, 41:5185–5192.
33. Endoh, T., Habe, H., Yoshida, T., Nojiri, H., and Omori, T., 2003, A CysB-regulated and sigma-54-dependent regulator, SfnR, is essential for dimethyl sulfone metabolism of *Pseudomonas putida* strain DS1. *Microbiology*, 149:991–1000.
34. Endoh, T., Kasuga, K., Horinouchi, M., Yoshida, T., Habe, H., Nojiri, H., and Omori, T., 2003, Characterization and identification of genes essential for dimethyl sulfide utilization in *Pseudomonas putida* strain DS1. *Appl. Microbiol. Biotechnol.*, 62:83–91.
35. Fitzgerald, J.W., Dodgson, K.S., and Payne, W.J., 1974, Induction of primary alkylsulphatases and metabolism of sodium hexan-1-yl sulphate by *Pseudomonas* C₁₂B. *Biochem. J.*, 138:63–69.
36. Fitzgerald, J.W. and Payne, W.J., 1972, The regulation of arylsulfatase activity in *Pseudomonas* C₁₂B. *Microbios*, 6:147–156.
37. Fitzgerald, J.W. and Scott, C.L., 1974, Utilization of choline-*O*-sulphate as a sulphur source for growth by a *Pseudomonas* isolate. *Microbios*, 10:121–131.
38. Fitzgerald, J.W., Stewart, G.J., and Kight Olliff, L., 1980, Regulation of primary alkylsulfatase induction in *Pseudomonas* C₁₂B: Concentration-dependent stimulation-inhibition by exogenous UTP and sodium acetate and inhibition by 1-hexanol. *Can. J. Microbiol.*, 26:1348–1355.
39. Foglino, M., Borne, F., Bally, M., Ball, G., and Patte, J.C., 1995, A direct sulphydrylation pathway is used for methionine biosynthesis in *Pseudomonas aeruginosa*. *Microbiology*, 141:431–439.
40. Gallardo, M.E., Ferrandez, A., De Lorenzo, V., Garcia, J.L., and Diaz, E., 1997, Designing recombinant *Pseudomonas* strains to enhance biodesulfurization. *J. Bacteriol.*, 179:7156–7160.
41. Gray, K.A., Pogrebinsky, O.S., Mrachko, G.T., Xi, L., Monticello, D.J., and Squires, C.H., 1996, Molecular mechanisms of biocatalytic desulfurization of fossil fuels. *Nature Biotechnol.*, 14:1705–1709.
42. Günther, E., Petruschka, L., and Herrmann, H., 1979, Reverse transsulfuration pathway in *Pseudomonas aeruginosa*. *Z. Allg. Mikrobiol.*, 19:439–442.
43. Harada, T., 1964, The formation of sulphatases in *Pseudomonas aeruginosa*. *Biochim. Biophys. Acta*, 81:193–196.
44. Haug, W., Schmidt, A., Nortemann, B., Hempel, D.C., Stolz, A., and Knackmuss, H.J., 1991, Mineralization of the sulfonated azo dye Mordant Yellow-3 by a 6-aminonaphthalene-2-sulfonate-degrading bacterial consortium. *Appl. Environ. Microbiol.*, 57:3144–3149.
45. Hsu, Y.C., 1965, Detergent-splitting enzyme from *Pseudomonas*. *Nature*, 207:385–388.
46. Hummerjohann, J., Kuttel, E., Quadroni, M., Ragaller, J., Leisinger, T., and Kertesz, M.A., 1998, Regulation of the sulfate starvation response in *Pseudomonas aeruginosa*: Role of cysteine biosynthetic intermediates. *Microbiology*, 144:1375–1386.
47. Hummerjohann, J., Laudenbach, S., Rétey, J., Leisinger, T., and Kertesz, M.A., 2000, The sulfur-regulated arylsulfatase gene cluster of *Pseudomonas aeruginosa*, a new member of the *cys* regulon. *J. Bacteriol.*, 182:2055–2058.

48. Imperato, T.J., Wong, C.G., Chen, L.J., and Bolt, R.J., 1977, Hydrolysis of lithocholate sulfate by *Pseudomonas aeruginosa*. *J. Bacteriol.*, 130:545–547.
49. Inoue, H., Inagaki, K., Eriguchi, S.I., Tamura, T., Esaki, N., Soda, K., and Tanaka, H., 1997, Molecular characterization of the *mde* operon involved in L-methionine catabolism of *Pseudomonas putida*. *J. Bacteriol.*, 179:3956–3962.
50. Inoue, H., Inagaki, K., Sugimoto, M., Esaki, N., Soda, K., and Tanaka, H., 1995, Structural analysis of the L-methionine gamma-lyase gene from *Pseudomonas putida*. *J. Biochem.*, 117:1120–1125.
51. Jansen, H.J., Hart, C.A., Rhodes, J.M., Saunders, J.R., and Smalley, J.W., 1999, A novel mucin-sulphatase activity found in *Burkholderia cepacia* and *Pseudomonas aeruginosa*. *J. Med. Microbiol.*, 48:551–557.
52. Junker, F., Leisinger, T., and Cook, A.M., 1994, 3-Sulphocatechol 2,3-dioxygenase and other dioxygenases (EC 1.13.11.2 and EC 1.14.12.-) in the degradative pathways of 2-amino-benzenesulphonic, benzenesulphonic and 4-toluenesulphonic acids in *Alcaligenes* sp. strain O-1. *Microbiology*, 140:1713–1722.
53. Kahnert, A. and Kertesz, M.A., 2000, Characterization of a sulfur-regulated oxygenative alkylsulfatase from *Pseudomonas putida* S-313. *J. Biol. Chem.*, 275:31661–31667.
54. Kahnert, A., Mirleau, P., Wait, R., and Kertesz, M.A., 2002, The LysR-type regulator SftR is involved in soil survival and sulfate ester metabolism in *Pseudomonas putida*. *Environ. Microbiol.*, 4:225–237.
55. Kahnert, A., Vermeij, P., Wietek, C., James, P., Leisinger, T., and Kertesz, M.A., 2000, The *ssu* locus plays a key role in organosulfur metabolism in *Pseudomonas putida* S-313. *J. Bacteriol.*, 182:2869–2878.
56. Kang, J.W., Kwon, A.R., Kim, D.H., and Choi, E.C., 2001, Cloning and sequencing of the *astA* gene encoding arylsulfate sulfotransferase from *Salmonella typhimurium*. *Biol. Pharm. Bull.*, 24:570–574.
57. Kertesz, M.A., 2001, Bacterial transporters for sulfate and organosulfur compounds. *Res. Microbiol.*, 152:279–290.
58. Kertesz, M.A., 1996, Desulfonation of aliphatic sulfonates by *Pseudomonas aeruginosa* PAO. *FEMS Microbiol. Lett.*, 137:221–225.
59. Kertesz, M.A., 1999, Riding the sulfur cycle—metabolism of sulfonates and sulfate esters in Gram-negative bacteria. *FEMS Microbiol. Rev.*, 24:135–175.
60. Kertesz, M.A., Kölbener, P., Stockinger, H., Beil, S., and Cook, A.M., 1994, Desulfonation of linear alkylbenzenesulfonate surfactants and related compounds by bacteria. *Appl. Environ. Microbiol.*, 60:2296–2303.
61. Kertesz, M.A., Leisinger, T., and Cook, A.M., 1993, Proteins induced by sulfate limitation in *Escherichia coli*, *Pseudomonas putida*, or *Staphylococcus aureus*. *J. Bacteriol.*, 175:1187–1190.
62. Kertesz, M.A., Schmidt-Larbig, K., and Wüest, T., 1999, A novel reduced flavin mononucleotide-dependent methanesulfonate sulfonase encoded by the sulfur-regulated *msu* operon of *Pseudomonas aeruginosa*. *J. Bacteriol.*, 181:1464–1473.
63. Key, B.D., Howell, R.D., and Criddle, C.S., 1998, Defluorination of organofluorine sulfur compounds by *Pseudomonas* sp. strain D2. *Environ. Sci. Technol.*, 32:2283–2287.
64. King, J.E., Jaouhari, R., and Quinn, J.P., 1997, The role of sulfoacetaldehyde sulfo-lyase in the mineralization of isethionate by an environmental *Acinetobacter* isolate. *Microbiology*, 7:2339–2343.
65. King, J.E. and Quinn, J.P., 1997, Metabolism of sulfoacetate by environmental *Aureobacterium* sp. and *Comamonas acidovorans* isolates. *Microbiology*, 12:3907–3912.
66. Kopriva, S., Buchert, T., Fritz, G., Suter, M., Benda, R.D., Schunemann, V., Koprivova, A., Schurmann, P., Trautwein, A.X., Kroneck, P.M.H., and Brunold, C., 2002, The presence of an

iron-sulfur cluster in adenosine 5'-phosphosulfate reductase separates organisms utilizing adenosine 5'-phosphosulfate and phosphoadenosine 5'-phosphosulfate for sulfate assimilation. *J. Biol. Chem.*, 277:21786–21791.

67. Kredich, N.M., 1996, Biosynthesis of cysteine. In F.C. Neidhardt, R. Curtiss, J.L. Ingraham, E.C.C. Lin, K.B. Low, B. Magasanik, W.S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (eds), *Escherichia coli* and *Salmonella typhimurium. Cellular and Molecular Biology*, 2nd edn, pp. 514–527. ASM Press, Washington.
68. Kropp, K.G. and Fedorak, P.M., 1998, A review of the occurrence, toxicity, and biodegradation of condensed thiophenes found in petroleum. *Can. J. Microbiol.*, 44:605–622.
69. Kwon, A.R., Yun, H.J., and Choi, E.C., 2001, Kinetic mechanism and identification of the active site tyrosine residue in *Enterobacter amnigenus* arylsulfate sulfotransferase. *Biochem. Biophys. Res. Commun.*, 285:526–529.
70. Lee, N.A. and Clark, D.P., 1993, A natural isolate of *Pseudomonas maltophilia* which degrades aromatic sulfonic acids. *FEMS Microbiol. Lett.*, 107:151–155.
71. Lisa, T.A., Casale, C.H., and Domenech, C.E., 1994, Cholinesterase, acid phosphatase, and phospholipase C of *Pseudomonas aeruginosa* under hyperosmotic conditions in a high phosphate medium. *Curr. Microbiol.*, 28:71–76.
72. Locher, H.H., Leisinger, T., and Cook, A.M., 1991, 4-Sulphobenzoate 3,4-dioxygenase. Purification and properties of a desulphonative two-component enzyme system from *Comamonas testosteroni* T-2. *Biochem. J.*, 274:833–842.
73. Lucas, J.J., Burchiel, S.W., and Segel, I.H., 1972, Choline sulfatase of *Pseudomonas aeruginosa*. *Arch. Biochem. Biophys.*, 153:664–672.
74. Lukatela, G., Krauss, N., Theis, K., Selmer, T., Gieselmann, V., von Figura, K., and Saenger, W., 1998, Crystal structure of human arylsulfatase A: The aldehyde function and the metal ion at the active site suggest a novel mechanism for sulfate ester hydrolysis. *Biochemistry*, 37:3654–3664.
75. Martelli, H.L. and Souza, S.M., 1970, Biochemistry of sulfonic compounds III. Formation of a two-carbon compound during the oxidation of sulfoacetate by a *Pseudomonas* strain. *Biochim. Biophys. Acta*, 208:110–115.
76. Matcham, G.W.J., Bartholomew, B., Dodgson, K.S., Fitzgerald, J.W., and Payne, W.J., 1977, Stereospecificity and complexity of microbial sulphohydrolases involved in the biodegradation of secondary alkylsulphate detergents. *FEMS Microbiol. Lett.*, 1:197–200.
77. McFarland, B.L., 1999, Bidesulfurization. *Curr. Opin. Microbiol.*, 2:257–264.
78. Miech, C., Dierks, T., Selmer, T., von Figura, K., and Schmidt, B., 1998, Arylsulfatase from *Klebsiella pneumoniae* carries a formylglycine generated from a serine. *J. Biol. Chem.*, 273:4835–4837.
79. Monticello, D.J., 2000, Bidesulfurization and the upgrading of petroleum distillates. *Curr. Opin. Biotechnol.*, 11:540–546.
80. Murooka, Y., Ishibashi, K., Yasumoto, M., Sasaki, M., Sugino, H., Azakami, H., and Yamashita, M., 1990, A sulfur- and tyramine-regulated *Klebsiella aerogenes* operon containing the arylsulfatase (*atsA*) gene and the *atsB* gene. *J. Bacteriol.*, 172:2131–2140.
81. Nelson, J.W., Tredgett, M.W., Sheehan, J.K., Thornton, D.J., Notman, D., and Govan, J.R.W., 1990, Mucinophilic and chemotactic properties of *Pseudomonas aeruginosa* in relation to pulmonary colonization in cystic fibrosis. *Infect. Immun.*, 58:1489–1495.
82. Nelson, K.E., Weinell, C., Paulsen, I.T., Dodson, R.J., Hilbert, H., dos Santos, V., Fouts, D.E., Gill, S.R., Pop, M., Holmes, M., Brinkac, L., Beanan, M., DeBoy, R.T., Daugherty, S., Kolonay, J., Madupu, R., Nelson, W., White, O., Peterson, J., Khouri, H., Hance, I., Lee, P.C., Holtzapple, E., Scanlan, D., Tran, K., Moazzes, A., Utterback, T., Rizzo, M., Lee, K., Kosack, D., Moestl, D., Wedler, H., Lauber, J., Stjepandic, D., Hoheisel, J., Straetz, M., Heim, S., Kiewitz, C., Eisen, J., Timmis, K.N., Dusterhoft, A., Tummeler, B., and Fraser, C.M.,

- 2002, Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ. Microbiol.*, 4:799–808.
83. Ochsner, U.A. and Vasil, M.L., 1996, Gene repression by the ferric uptake regulator in *Pseudomonas aeruginosa*: Cycle selection of iron-regulated genes. *Proc. Natl. Acad. Sci. USA*, 93:4409–4414.
84. Ohe, T., Ohmoto, T., Kobayashi, Y., Sato, A., and Watanabe, Y., 1990, Metabolism of naphthalenesulfonic acids by *Pseudomonas* sp. TA-2. *Agric. Biol. Chem.*, 54:669–675.
85. Ohe, T. and Watanabe, Y., 1986, Degradation of 2-naphthylamine-1-sulfonic acid by *Pseudomonas* strain TA-1. *Agric. Biol. Chem.*, 50:1419–1426.
86. Ohe, T. and Watanabe, Y., 1988, Microbial degradation of 1,6-naphthalenedisulfonic and 2,6-naphthalenedisulfonic acid by *Pseudomonas* sp. DS-1. *Agric. Biol. Chem.*, 52:2409–2414.
87. Osteras, M., Boncompagni, E., Vincent, N., Poggi, M.C., and Le Rudulier, D., 1998, Presence of a gene encoding choline sulfatase in *Sinorhizobium meliloti* bet operon: Choline-*O*-sulfate is metabolized into glycine betaine. *Proc. Natl. Acad. Sci. USA*, 95:11394–11399.
88. Pao, S.S., Paulsen, I.T., and Saier, M.H., Jr, 1998, Major facilitator superfamily. *Microbiol. Mol. Biol. Rev.*, 62:1–34.
89. Payne, W.J. and Faisal, V.E., 1963, Bacterial utilization of dodecylsulfate and dodecyl benzenesulfonate. *Appl. Microbiol.*, 11:339–344.
90. Proksova, M., Augustin, J., and Vrbanova, A., 1997, Enrichment, isolation and characterization of dialkyl sulfosuccinate degrading bacteria *Comamonas terrigena* N3H and *Comamonas terrigena* N1C. *Folia Microbiol.*, 42:635–639.
91. Quadroni, M., James, P., Dainese-Hatt, P., and Kertesz, M.A., 1999, Proteome mapping, mass spectrometric sequencing and reverse transcriptase-PCR for characterisation of the sulfate starvation-induced response in *Pseudomonas aeruginosa* PAO1. *Eur. J. Biochem.*, 266:986–996.
92. Quadroni, M., Staudenmann, W., Kertesz, M., and James, P., 1996, Analysis of global responses by protein and peptide fingerprinting of proteins isolated by two-dimensional gel electrophoresis: Application to the sulfate-starvation response of *Escherichia coli*. *Eur. J. Biochem.*, 239:773–781.
93. Quick, A., Russell, N.J., Hales, S.G., and White, G.F., 1994, Biodegradation of sulphosuccinate: Direct desulphonation of a secondary sulphonate. *Microbiology*, 140:2991–2998.
94. Rammler, D.H., Grado, C., and Fowler, L.R., 1964, Sulfur metabolism of *Aerobacter aerogenes* 1. A repressible sulfatase. *Biochemistry*, 3:224–230.
95. Ramphal, R., Carnoy, C., Fiebre, S., Michalski, J.C., Houdret, N., Lamblin, G., Strecker, G., and Roussel, P., 1991, *Pseudomonas aeruginosa* recognizes carbohydrate chains containing type 1 (Gal-Beta-1-3GlcNac) or Type-2 (Gal-Beta-1-4GlcNac) disaccharide units. *Infect. Immun.*, 59:700–704.
96. Reichenbecher, W., Kelly, D.P., and Murrell, J.C., 1999, Desulfonation of propanesulfonic acid by *Comamonas acidovorans* strain P53: Evidence for an alkanesulfonate sulfonataase and an atypical sulfite dehydrogenase. *Arch. Microbiol.*, 172:387–392.
97. Reichenbecher, W. and Murrell, J.C., 1999, Linear alkanesulfonates as carbon and energy sources for gram-positive and gram-negative bacteria. *Arch. Microbiol.*, 171:430–438.
98. Rocha, E.P. C., Sekowska, A., and Danchin, A., 2000, Sulphur islands in the *Escherichia coli* genome: Markers of the cell's architecture? *FEBS Lett.*, 476:8–11.
99. Ruff, J., Hitzler, T., Rein, U., Ritter, A., and Cook, A.M., 1999, Bioavailability of water-polluting sulfonoaromatic compounds. *Appl. Microbiol. Biotechnol.*, 52:446–450.
100. Satishchandran, C., Hickman, Y.N., and Markham, G.D., 1992, Characterization of the phosphorylated enzyme intermediate formed in the adenosine 5'-phosphosulfate kinase reaction. *Biochemistry*, 31:11684–11688.

101. Schleheck, D., Dong, W.B., Denger, K., Heinzle, E., and Cook, A.M., 2000, An alpha-proteobacterium converts linear alkylbenzenesulfonate surfactants into sulfophenylcarboxylates and linear alkyldiphenyletherdisulfonate surfactants into sulfodiphenylethercarboxylates. *Appl. Environ. Microbiol.*, 66:1911–1916.
102. Schulz, S., Dong, W.B., Groth, U., and Cook, A.M., 2000, Enantiomeric degradation of 2-(4-sulfophenyl) butyrate via 4-sulfocatechol in *Delftia acidovorans* SPB1. *Appl. Environ. Microbiol.*, 66:1905–1910.
103. Seitz, A.P., Leadbetter, E.R., and Godchaux, W., 1993, Utilization of sulfonates as sole sulfur source by soil bacteria including *Comamonas acidovorans*. *Arch. Microbiol.*, 159:440–444.
104. Shan, X.Q., Chen, B., Zhang, T.H., Li, F.L., Wen, B., and Qian, J., 1997, Relationship between sulfur speciation in soils and plant availability. *Sci. Total Environ.*, 199:237–246.
105. Shaw, D.J., Dodgson, K.S., and White, G.F., 1980, Substrate specificity and other properties of the inducible S3 secondary alkylsulphohydrolase purified from the detergent-degrading bacterium *Pseudomonas* C₁₂B. *Biochem. J.*, 187:181–196.
106. Shimamoto, G. and Berk, R.S., 1979, Catabolism of taurine in *Pseudomonas aeruginosa*. *Biochim. Biophys. Acta*, 569:287–292.
107. Shimamoto, G. and Berk, R.S., 1980, Taurine catabolism II. Biochemical and genetic evidence for sulfoacetaldehyde sulfolase involvement. *Biochim. Biophys. Acta*, 632:121–130.
108. Smith, L.T., Pocard, J.A., Bernard, T., and Le Rudulier, D., 1988, Osmotic control of glycine betaine biosynthesis and degradation in *Rhizobium meliloti*. *J. Bacteriol.*, 170:3142–3149.
109. Stolz, A., 2001, Basic and applied aspects in the microbial degradation of azo dyes. *Appl. Microbiol. Biotechnol.*, 56:69–80.
110. Stolz, A., 1999, Degradation of substituted naphthalenesulfonic acids by *Sphingomonas xenophaga* BN6. *J. Ind. Microbiol. Biotechnol.*, 23:391–399.
111. Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrenner, P., Hickey, M.J., Brinkman, F.S.L., Hufnagle, W.O., Kowalik, D.J., Lagrou, M., Garber, R.L., Goltry, L., Tolentino, E., Westbrook-Wadman, S., Yuan, Y., Brody, L.L., Coulter, S.N., Folger, K.R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G.K.S., Wu, Z., Paulsen, I.T., Reizer, J., Saier, M.H., Hancock, R.E.W., Lory, S., and Olson, M.V., 2000, Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature*, 406:959–964.
112. Strous, G.J. and Dekker, J., 1992, Mucin-type glycoproteins. *Crit. Rev. Biochem. Mol. Biol.*, 27:57–92.
113. Szameit, C., Miech, C., Balleininger, M., Schmidt, B., von Figura, K., and Dierks, T., 1999, The iron sulfur protein AtsB is required for posttranslational formation of formylglycine in the *Klebsiella* sulfatase. *J. Biol. Chem.*, 274:15375–15381.
114. Tazuke, Y., Matsuda, K., Adachi, K., and Tsukada, Y., 1998, Purification and properties of a novel sulfatase from *Pseudomonas testosteroni* that hydrolyzed 3 beta-hydroxy-5-cholenoic acid 3-sulfate. *Biosci. Biotechnol. Biochem.*, 62:1739–1744.
115. Tazuke, Y., Matsuda, K., Adachi, K., and Tsukada, Y., 1994, Purification and properties of bile acid sulfate sulfatase from *Pseudomonas testosteroni*. *Biosci. Biotechnol. Biochem.*, 58:889–894.
116. Tazuke, Y., Matsuda, K., Okada, S., and Tsukada, Y., 1992, A novel sulfatase from *Pseudomonas testosteroni* hydrolyzing lithocholic acid sulfate. *Biosci. Biotechnol. Biochem.*, 56:1584–1588.
117. Thomas, O.R.T., Matts, P.J., and White, G.F., 1988, Localization of electron microscopy of alkylsulfatases in bacterial cells. *J. Gen. Microbiol.*, 134:1229–1236.
118. Thyse, G.J.E. and Wanders, T.H., 1972, Degradation of *n*-alkane-1-sulfonates by *Pseudomonas*. *Antonie van Leeuwenhoek*, 38:53–63.
119. Thyse, G.J.E. and Wanders, T.H., 1974, Initial steps in the degradation of *n*-alkane-1-sulphonates by *Pseudomonas*. *Antonie van Leeuwenhoek*, 40:25–37.

120. Tralau, T., Cook, A.M., and Ruff, J., 2001, Map of the IncP1 beta plasmid pTSA encoding the widespread genes (*tsa*) for *p*-toluenesulfonate degradation in *Comamonas testosteroni* T-2. *Appl. Environ. Microbiol.*, 67:1508–1516.
121. Tralau, T., Mampel, J., Cook, A.M., and Ruff, J., 2003, Characterization of TsaR, an oxygen-sensitive LysR-type regulator for the degradation of *p*-toluenesulfonate in *Comamonas testosteroni* T-2. *Appl. Environ. Microbiol.*, 69:2298–2305.
122. Tralau, T., Wietek, C., and Kertesz, M.A., 2003, Desulfonation of aliphatic and aromatic sulfonates in *Pseudomonas putida* S-313 by a sulfate starvation-induced monooxygenase system, unpublished results.
123. Tsai, H.H., Hart, C.A., and Rhodes, J.M., 1991, Production of mucin degrading sulphatases and glycosidases by *Bacteroides thetaiotaomicron*. *Lett. Appl. Microbiol.*, 13:97–101.
124. Vairavamurthy, M.A., Maletic, D., Wang, S.K., Manowitz, B., Eglinton, T., and Lyons, T., 1997, Characterization of sulfur-containing functional groups in sedimentary humic substances by X-ray absorption near-edge structure spectroscopy. *Energy Fuels*, 11:546–553.
125. Vairavamurthy, M.A., Zhou, W., Eglinton, T., and Manowitz, B., 1994, Sulfonates: A new class of organic sulfur compounds in marine sediments. *Geochim. Cosmochim. Acta*, 58:4681–4687.
126. van der Ploeg, J.R., Cummings, N.J., Leisinger, T., and Connerton, I.F., 1998, *Bacillus subtilis* genes for the utilization of sulfur from aliphatic sulfonates. *Microbiology*, 144:2555–2561.
127. van der Ploeg, J.R., Eichhorn, E., and Leisinger, T., 2001, Sulfonate-sulfur metabolism and its regulation in *Escherichia coli*. *Arch. Microbiol.*, 176:1–8.
128. van der Ploeg, J.R., Iwanicka-Nowicka, R., Bykowski, T., Hryniewicz, M., and Leisinger, T., 1999, The Cbl-regulated *ssuEADCB* gene cluster is required for aliphatic sulfonate-sulfur utilization in *Escherichia coli*. *J. Biol. Chem.*, 174:29358–29365.
129. van der Ploeg, J.R., Iwanicka-Nowicka, R., Kertesz, M.A., Leisinger, T., and Hryniewicz, M.M., 1997, Involvement of CysB and Cbl regulatory proteins in expression of the *tauABCD* operon and other sulfate starvation-inducible genes in *Escherichia coli*. *J. Bacteriol.*, 179:7671–7678.
130. Vermeij, P. and Kertesz, M.A., 1999, Pathways of assimilative sulfur metabolism in *Pseudomonas putida*. *J. Bacteriol.*, 181:5833–5837.
131. Vermeij, P., Wietek, C., Kahnert, A., Wüest, T., and Kertesz, M.A., 1999, Genetic organization of sulfur-controlled aryl desulfonation in *Pseudomonas putida* S-313. *Mol. Microbiol.*, 32:913–926.
132. von Figura, K., Schmidt, B., Selmer, T., and Dierks, T., 1998, A novel protein modification generating an aldehyde group in sulfatases: Its role in catalysis and disease. *Bioessays*, 20:505–510.
133. Watwood, M.E., Fitzgerald, J.W., and Gosz, J.R., 1986, Sulfur processing in forest soil and litter along an elevational and vegetative gradient. *Can. J. For. Res.*, 16:689–695.
134. Wehnert, M., Günther, E., and Herrmann, H., 1975, Vitamin B₁₂-dependent methionine biosynthesis in *Pseudomonas aeruginosa*. *Z. Allg. Mikrobiol.*, 15:281–286.
135. White, G.F., Russell, N.J., and Day, M.J., 1985, A survey of sodium dodecyl-sulfate (SDS) resistance and alkylsulfatase production in bacteria from clean and polluted river sites. *Environ. Poll. Series A*, 37:1–11.
136. Wright, D.P., Knight, C.G., Parker, S.G., Christie, D.L., and Robertson, A.M., 2000, Cloning of a mucin-desulfating sulfatase gene from *Prevotella* strain RS2 and its expression using a *Bacteroides* recombinant system. *J. Bacteriol.*, 182:3002–3007.
137. Zhao, Q.X. and Poole, K., 2000, A second *tonB* gene in *Pseudomonas aeruginosa* is linked to the *exbB* and *exbD* genes. *FEMS Microbiol. Lett.*, 184:127–132.
138. Zürrer, D., Cook, A.M., and Leisinger, T., 1987, Microbial desulfonation of substituted naphthalenesulfonic acids and benzenesulfonic acids. *Appl. Environ. Microbiol.*, 53:1459–1463.

RING-CLEAVAGE DIOXYGENASES

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1. INTRODUCTION

Pseudomonads have an exceptional ability to utilize aromatic compounds as sole source of energy and carbon. This capability is critical to maintaining the global carbon cycle. Aromatic compounds are planar, fully conjugated, ring-shaped molecules possessing $(4n + 2)\pi$ electrons where n is a nonnegative integer (Hückel's rule)⁹³. Formed by a variety of biogeochemical processes, these compounds are widely distributed in nature, and range in size from low-molecular-mass compounds, such as phenols, to biopolymers, such as lignin. Indeed, lignin is the second most abundant polymer in nature after cellulose². Aromatic compounds are exceptionally stable due to the delocalization of their π orbitals (resonance structure). This property has contributed to the wide spread production and usage of natural and non-natural aromatic compounds for a variety of industrial applications, as well as the distribution of stable, nonnatural compounds in the environment.

Microorganisms have evolved diverse catabolic pathways to degrade aromatic compounds, including anaerobic and aerobic strategies. Regardless of the specific catabolic strategy, these pathways involve two key steps: the

activation of the thermodynamically stable benzene ring, and its subsequent cleavage. In aerobic microbial degradation, oxygenases activate the benzene ring by catalyzing the incorporation of oxygen-containing substituents. The critical step of ring fission is then catalyzed by ring-cleaving dioxygenases^{29, 30, 57}, which are the subject of this chapter. Much of our understanding of these dioxygenases is derived from studies involving pseudomonads. Indeed, most of these enzymes were initially identified in pseudomonads^{60, 80, 106, 132, 134}, and studies on these microorganisms and their enzymes have continued to provide critical insights into our understanding of ring-cleaving dioxygenases.

This chapter covers metalloenzymes that are involved in the oxidative ring-cleavage of aromatic compounds. Two other classes of ring-cleaving enzymes that are involved in the aerobic catabolism of aromatic compounds but that are not covered in this chapter are the cofactor-less dioxygenases that catalyze the 2,4-cleavage of 3-hydroxy-4-quinolones and hydrolytic enzymes. The former possess a serine hydrolase fold and were recently reviewed by Fetzner⁴³. The latter are part of the CoA-driven reductive pathways traditionally associated with the anaerobic degradation of aromatic compounds. However, it is now clear that some of these pathways function under aerobic conditions^{49, 98, 162}.

In addition to their fundamental significance, ring-cleaving dioxygenases are of interest due to their potential utility in the degradation of environmental pollutants such as polychlorinated biphenyls (PCBs). As discussed in Chapters 18 and 19, pseudomonads and other bacteria are able to transform xenobiotic compounds that structurally resemble naturally occurring growth substrates. These organisms and their catabolic pathways are of interest for bioremedial applications. In many instances, the ring-cleaving dioxygenase of the relevant pathway is an important determinant of the specificity of the pathway. For example, the inactivation and/or specificity of these enzymes inhibit the simultaneous degradation of chloro- and alkylaromatics¹²¹ as well as the degradation of certain PCB congeners³¹. Several strategies to improve the degradation of such recalcitrant compounds have been described¹³⁷.

2. OCCURRENCE WITHIN CATABOLIC PATHWAYS

In principle, microorganisms utilize a distinct catabolic pathway to degrade each type of aromatic compound. Nevertheless, the aerobic catabolism of these compounds usually proceeds via one of four intermediates (Figure 1): catechol, protocatechuate, gentisate or hydroquinone (benzene-1,4-diol). Related compounds, such as homoprotocatechuate, dihydroxyphenyl

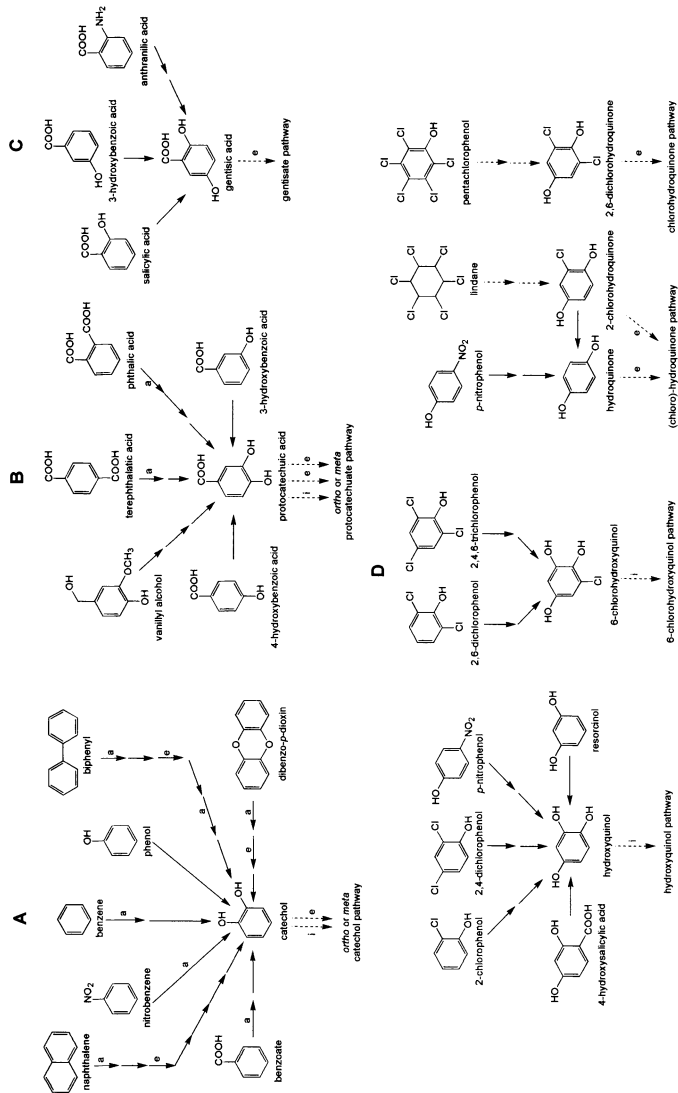


Figure 1. Pathways for the catabolism of aromatic compounds leading to one of four intermediates: (A) catechol; (B) protocatechuic acid; (C) gentisic acid; and (D) (chloro)hydroxyquinol and (chloro)hydroquinone. Each solid arrow indicates a single enzyme-catalyzed reaction. Reactions designated by “a” are catalyzed by a ring-hydroxylating dioxygenase (see Chapter 22). Reactions designated by “i” are catalyzed by an intradiol dioxygenase and reactions designated by “e” are catalyzed by an extradiol or extradiol-type dioxygenase. The summarized pathways are representative of those involving one of the four ring-cleavage intermediates. Several other pathways have been reported but could not be described in detail in this chapter.

propionates and homogentisate, also occur as intermediates. In addition, at least two other types of compounds have been identified as substrates for ring cleavage reactions: salicylate and 2-aminophenol. These various intermediates occur in the degradation of monocyclic compounds, as described in the subsections below. Compounds containing more than one aromatic ring are degraded via iterations of the strategies used to degrade monocyclic compounds (Figure 1A).

The ring cleavage of catecholic compounds is performed by enzymes from one of two distinct classes: intradiol and extradiol dioxygenases (Figure 2)⁵⁶. Intradiol dioxygenases utilize non-heme Fe(III) to cleave the aromatic nucleus *ortho* to (between) the hydroxyl substituents. In contrast, extradiol dioxygenases utilize non-heme Fe(II) to cleave the aromatic nucleus *meta* (adjacent) to the hydroxyl substituents. Interestingly, a few Mn(II)-dependent extradiol dioxygenases with strong sequence similarity to Fe(II) counterparts have also been reported^{59, 116}, as has an Mg(II)-containing enzyme of unknown phylogeny⁵⁰. Although the distinctions between intradiol and extradiol dioxygenases may appear to be minor, they are in fact a manifestation of enzymes that have completely different structures and utilize different catalytic mechanisms (for recent reviews see refs [16] and [129]).

One notable difference between intradiol and extradiol enzymes is that the former appears to only cleave substrates possessing vicinal hydroxyl groups. In addition to catechol, the only known substrates for intradiol enzymes are protocatechuate and 2-hydroxyquinol (1,2,4-trihydroxybenzene), which are essentially substituted catechols. In contrast, not all aromatic compounds that are subject to extradiol-type cleavage possess vicinal hydroxyl groups. Non-catecholic compounds that are subject to extradiol-type cleavage include the other intermediates mentioned above: gentisate, hydroquinone, salicylate and 2-aminophenol. In comparison to the substrates of typical extradiol dioxygenases, these compounds are either dihydroxylated in the *para* positions and/or possess a carboxylate or an amino group in place of the second hydroxyl group. The products of the ring cleavage of each of the four “major” intermediates listed at the outset of this section, and their further transformation to intermediates of the tricarboxylic acid cycle are summarized in Figure 2. As discussed in Section 9, the absolute requirement of intradiol dioxygenases for substrates possessing vicinal hydroxyl groups is consistent with the proposed mechanistic differences between intradiol and extradiol enzymes.

A second difference between intradiol and extradiol enzymes is that the former generally cleave catechols possessing electron-withdrawing substituents. In contrast, extradiol enzymes cleave catechols possessing electron-donating substituents. There are nevertheless examples of extradiol enzymes that cleave halogenated substrates. Thus, in the catabolism of chlorobenzene by *Pseudomonas putida* GJ31, a chlorocatechol 2,3-dioxygenase (CC23O_{GJ31})

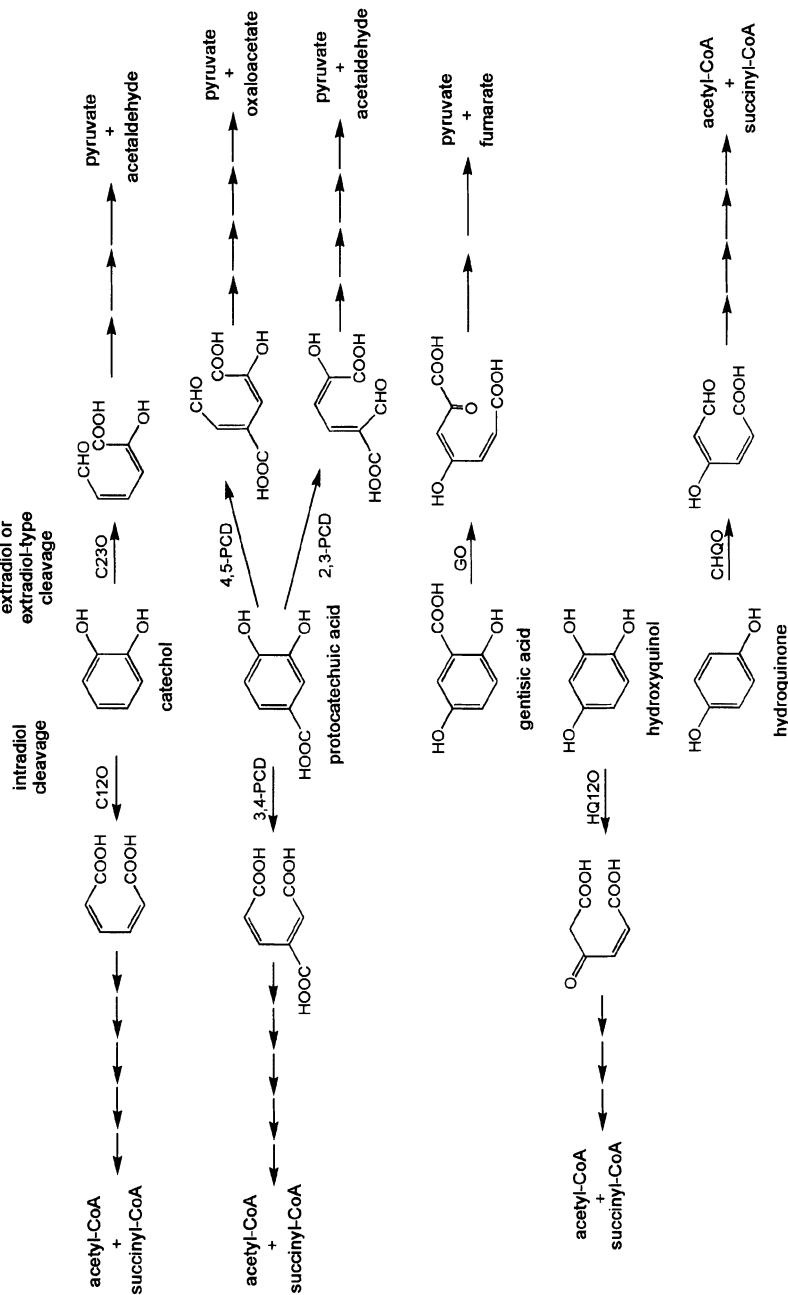


Figure 2. Products of ring-cleavage dioxygenase-catalyzed reactions. Each arrow indicates a single enzyme-catalyzed reaction. Acetaldehyde enters the tricarboxylic acid cycle as acetyl-CoA (adapted from refs [29] and [30]).

catalyzes the cleavage of 3-chlorocatechol⁸⁸. 3-Chlorocatechol may also be cleaved in a 1,6-fashion¹¹⁹. However, the physiological relevance of this reaction is unclear.

2.1. Catechols

In many respects, catechol dioxygenases are the prototypical ring-cleavage enzymes. Thus, a metapyrocatechase, or catechol 2,3-dioxygenase (C23O) from a pseudomonad was the first identified extradiol dioxygenase⁸⁰. Studies on C23O were the first to demonstrate these dioxygenases require ferrous iron¹⁰² and that they utilize an ordered, ternary complex mechanism⁶⁶. Similarly, one of the first identified intradiol dioxygenases was a catechol 1,2-dioxygenase (C12O) involved in the catabolism of benzoates⁶⁰.

Catechol occurs in the degradation of benzene⁵¹, benzoate¹⁰⁰, phenol⁶⁷ and derivatives thereof. The latter include alkylated, nitrosylated and chlorinated derivatives. Catecholic intermediates that arise in the degradation of polycyclic aromatics may be considered as alkylated catechols. Accordingly, intermediates such as 1,2-dihydroxynaphthalene and 2,3-dihydroxybiphenyl, which occur in the degradation of naphthalene and biphenyl respectively^{21, 72}, are subject to extradiol cleavage.

2.2. Protocatechuates

Protocatechuate occurs in the degradation of hydroxybenzoates^{27, 90}, phthalates⁷⁵ and vanillyl alcohols^{91, 113}. Protocatechuate is subject to three different modes of cleavage: protocatechuate 3,4-dioxygenase (3,4-PCD)¹³² catalyzes intradiol cleavage, whereas protocatechuate 2,3-dioxygenase (2,3-PCD)¹⁵⁸ and protocatechuate 4,5-dioxygenase (4,5-PCD)¹⁰⁶ catalyze each of two different modes of extradiol cleavage.

The related compound, homoprotocatechuate (3,4-dihydroxyphenylacetate), is cleaved in an extradiol fashion by homoprotocatechuate 2,3-dioxygenase (HPCD) in the degradation of 4-chlorophenylacetate⁷⁷ and 3- and 4-hydroxyphenylacetate²⁶. Related HPCDs utilizing Fe(II) and Mn(II) have been isolated from *Brevibacterium fuscum*¹⁵³ and *Arthrobacter globiformis* CM-2¹⁵⁶, respectively. A HPCD that utilizes Mg(II) has also been purified⁵⁰. However, the phylogenetic relationship of this enzyme to other extradiol dioxygenases is not known.

2.3. Gentisates and Salicylates

The ring cleavage of gentisate is catalyzed by gentisate 1,2-dioxygenase (GO)^{28, 58}, an extradiol-type dioxygenase. Gentisate has been identified as an

intermediate in the catabolism of salicylate⁵², 3-hydroxybenzoic acid⁶⁵ and anthranilate¹⁹. As noted in Section 2.6, an important derivative of gentisate, homogentisate, occurs in the catabolism of phenylalanine and tyrosine.

A GO has been described that can also cleave salicylate⁶⁴. This is an interesting reaction. However, it is unclear whether there are any dioxygenases that preferentially utilize salicylate. Related compounds that are subject to extradiol-type cleavage include 5-aminosalicylate¹³³ and 1-hydroxy-2-naphthoate, which is involved in the catabolism of phenanthrene⁷⁰. 1-Hydroxy-2-naphthoate dioxygenase did not detectably cleave either salicylate or gentisate.

2.4. Hydroxyquinols and Hydroquinones

Hydroxyquinols and hydroquinones are 1,4-dihydroxybenzenes (Figure 1D). 2-Hydroxyquinols and its chlorinated derivatives are cleaved by 2-hydroxyquinol 1,2-dioxygenase (HQ12O), an intradiol enzyme. This enzyme occurs in the catabolism of *p*-nitrophenol⁷¹, resorcinol⁴⁷, and 4-hydroxysalicylic acid⁶. The related 6-chloro-HQ12O occurs in the catabolism of 2,6-dichlorophenol¹⁶³ and 2,4,6-trichlorophenol¹⁶³. It is unclear whether HQ12O or 6-Cl HQ12O is involved in the catabolism of 2-chlorophenol and 2,4-dichlorophenol¹⁶³. Hydroquinones are cleaved by an extradiol-type enzyme, hydroquinone dioxygenase (HQO). This enzyme is involved in the degradation of *p*-nitrophenol²⁴, pentachlorophenol¹⁶⁰ and lindane⁹⁷, which is aromaticized as it is dehalogenated.

2.5. 2-Aminophenols

2-Aminophenols are cleaved by Fe(II)-dependent extradiol-type dioxygenases. 2-Aminophenol 1,6-dioxygenases (APDs) are involved in the degradation of nitrobenzene⁸⁵ and 2-aminophenol¹³⁶. A notable derivative of 2-aminophenol is 3-hydroxyanthranilate. This compound is an intermediate in the catabolism of 2-nitrobenzoate by a pseudomonad⁹⁹ as well as in the catabolism of tryptophan (Section 2.6).

2.6. Aromatic Amino Acids

Ring-cleavage reactions are also involved in the aerobic catabolism of aromatic amino acids. Thus, homogentisate dioxygenase (HGO) is involved in the catabolism of phenylalanine and tyrosine, and 3-hydroxyanthranilate dioxygenase (HAD) is involved in the catabolism of tryptophan in the kynurenine pathway. These dioxygenases, which are extradiol-type enzymes, occur in organisms from *Pseudomonas* to man. In humans, HGO and HAD are associated with the genetic disorders alkaptonuria and Huntington's disease, respectively^{83, 125}.

3. CLASSIFICATION OF RING-CLEAVING DIOXYGENASES

Intradiol and extradiol enzymes share no significant sequence or structural similarities and thus belong to evolutionary distinct classes of proteins. Sequence and structural analyses further indicate that all intradiol dioxygenases characterized to date belong to a single evolutionary lineage. Thus, despite their different subunit compositions, the catalytic domains of 3,4-PCD and C12O share a common structural fold¹⁴⁹. Moreover, these enzymes share key conserved residues with the HQ12O, including the four endogenous iron ligands³³ (see Section 5).

In contrast to the intradiol dioxygenases, extradiol and extradiol-type dioxygenases belong to at least three evolutionarily independent families^{38, 41} (Table 1). Type I extradiol dioxygenases belong to the vicinal oxygen chelate superfamily^{7, 48}. Type I extradiol dioxygenase includes two-domain and one-domain enzymes. The former are exemplified by 2,3-dihydroxybiphenyl 1,2-dioxygenase (DHBD) from *Burkholderia* sp. LB400 (DHBD_{LB400}), C23O_{mt2}, HPCD_{Bfu} and chlorohydroquinone dioxygenase (CHQO)^{97, 160}. One-domain enzymes are exemplified by two DHBD isozymes from *Rhodococcus globerulus* P6 (DHBD_{p6}-II and DHBD_{p6}-III). Type II extradiol dioxygenases include enzymes consisting of one (e.g., 2,3-dihydroxyphenylpropionate 1,2-dioxygenase)¹³⁰ or two different subunits. As discussed in Section 4.2, there are examples of type II enzymes in which the two different subunits are related (APD_{JS45})³⁴ and there are examples in which the two subunits appear to be unrelated (protocatechuate 4,5-dioxygenase [4,5-PCD])¹³⁵. A third family of extradiol-type dioxygenases belong to the cupin superfamily³⁸. Dioxygenases belonging to this superfamily include GO, 1-hydroxy-2-naphthoate dioxygenase, HGO and HAD.

As is evident from Table 1, there is no strict correlation between substrate specificity and evolutionary origin in extradiol dioxygenases. Thus, there are examples of type I and II HPCDs, as well as type I and II C23Os. It is nonetheless noted that the substrate specificity of most ring-cleavage enzymes has not been well investigated. This shortcoming is particularly evident in cases in which multiple DHBDs have been identified in a single strain. In many instances, such enzymes were identified on the basis of plate assays performed using a single substrate at a single concentration. Proper investigation of these enzymes may well reveal that their substrate specificities are quite different from one another.

The respective superfamilies to which type I and III extradiol dioxygenases belong are discussed below. The type II extradiol dioxygenases are not discussed in this respect as they have not been classified in a superfamily. The structural features of each type of extradiol dioxygenase are discussed in Section 4.

Table 1. Families of ring-cleavage enzymes based on structural folds.

Type	Superfamily	Prototypic members	Subunit ^a
Intradiol		3,4-PCD _{B-10} C12O _{ADP1} HQ12O	(αβ) ₁₂ α ₂ α ₂
Extradiol			
I	Vicinal oxygen chelate	DHBD _{LB400} C23O _{mt2} HPCD _{Bfu} CHQO DHBD _{p6-II}	α ₈ α ₄ α _x α _x α ₆
II	unknown	2,3-dihydroxyphenylpropionate 1,2-dioxygenase _{Eco} HPCD _{EcoC} C23O _{JMP222-I} 4,5-PCD _{SYK6} APD _{JS45} GO	α ₄ α _x α _x α ₂ β ₂ α ₂ β ₂ α ₄
III	Cupin	1-hydroxy-2-naphthoate dioxygenase _{KP7}	α ₆

3,4-PCD_{B-10}, protocatechuate 3,4-dioxygenase from *P. putida* B-10¹⁰⁴; C12O_{ADP1}, catechol 1,2-dioxygenase from *Acinetobacter* sp. ADP1¹⁴⁹; HQ12O, hydroxyquinol 1,2-dioxygenase from *Nocardioides simplex* 3E¹²; DHBD_{LB400}, 2,3-dihydroxybiphenyl 1,2-dioxygenase from *Burkholderia* sp. LB400⁵⁴; C23O_{mt2}, catechol 2,3-dioxygenase from *P. putida* mt-2⁷⁶; HPCD_{Bfu}, homoprotocatechuate 2,3-dioxygenase from *B. fuscum*¹⁵³; CHQO, chlorohydroquinone dioxygenase; DHBD_{p6-II}, 2,3-dihydroxybiphenyl 1,2-dioxygenase II from *R. globerulus* P6⁹; 2,3-dihydroxyphenylpropionate 1,2-dioxygenase from *Escherichia coli*¹⁷; HPCD_{EcoC}, homoprotocatechuate 2,3-dioxygenase from *E. coli* C¹²²; C23O_{JMP222-I}, catechol 2,3-dioxygenase I from *Alcaligenes eutrophus* JMP222⁷³; 4,5-PCD_{SYK6}, protocatechuate 4,5-dioxygenase from *Sphingomonas paucimobilis* SYK-6¹³⁵; APD_{JS45}, 2-aminophenol 1,6-dioxygenase from *Pseudomonas pseudoalcaligenes* JS45³⁴; GO, gentisate dioxygenase from *Pseudomonas testosteroni*⁵⁸; 1-hydroxy-2-naphthoate dioxygenase from *Nocardioides* sp. KP7⁷⁰.

^a An “x” indicates that the oligomeric state of the enzyme is not known.

3.1. Type I Extradiol Dioxygenases and the Vicinal Oxygen Chelate Superfamily

The common structural fold of the vicinal-oxygen-chelate superfamily^{7, 48} is a module consisting of four β strands and one α helix that occur in the following sequence: βαβββ. Each structural domain of the type I extradiol dioxygenases contains two copies of this module. Phylogenetic analyses indicate that these dioxygenases share a common one-domain ancestor; the evolution of type I extradiol dioxygenases therefore appears to have involved two duplication events followed by the divergence of one- and two-domain enzymes. Subsequent divergence among the two-domain dioxygenases has resulted in several families, at least two of which are based on substrate

preference^{41, 56}. DHBD_{LB400} and DHBD_{P6-I} belong to a family with a preference for bicyclic substrates. In contrast, C23O_{mt2} belongs to a family with a preference for monocyclic substrates.

Most members of the vicinal-oxygen-chelate superfamily utilize a divalent metal ion to catalyze a reaction involving direct metal ion chelation by vicinal oxygens of the substrate or an intermediate in the reaction. The members of the superfamily identified to date are: (a) type I extradiol dioxygenases; (b) 4-hydroxyphenylpyruvate dioxygenase¹²⁷ (EC 1.13.11.27); (c) glyoxalases I²⁰ (EC 4.4.1.5); (d) fosfomycin resistance proteins¹³; and (e) methylmalonyl-CoA epimerases⁹² (EC 5.1.99.1). The metal ion requirement of

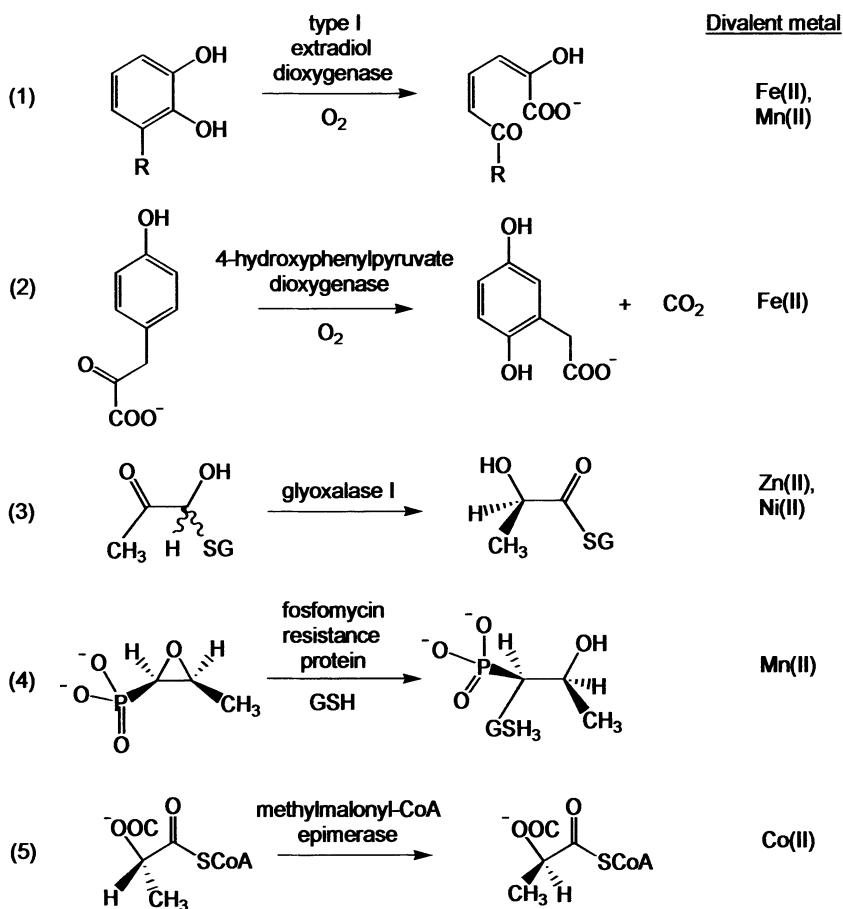


Figure 3. Reactions catalyzed by members of the vicinal oxygen chelate superfamily (adapted from ref. [48]).

these enzymes, and the reactions they catalyze, are summarized in Figure 3. To date, the one exception in the superfamily is the bleomycin resistance proteins (BRP)³⁷, which form a sixth family of the vicinal-oxygen-chelate superfamily. BRPs do not bind any metal ion and sequester bleomycin and related compounds without degrading or transforming them. It is thought that during the evolution of this protein, the divalent metal ion may have been shed in favor of a more hydrophobic cavity to accommodate the antibiotic. The use of the pseudosymmetric structure provided by the pair of $\beta\alpha\beta\beta$ modules offers a versatile template and structural flexibility when compared to the well-known barrel folds.

3.2. Type III Extradiol Dioxygenases and the Cupin Superfamily

GO, 1-hydroxy-2-naphthoate dioxygenase, HGO and HAD are part of the cupin superfamily³⁸. This superfamily is composed of proteins containing at least one domain with six antiparallel β -strands that form a β -barrel structure. Within this β -barrel are two distinct motifs. The first motif is composed of the first two β -strands and the second, of the last two β -strands. One of the major differences between the various classes of cupins involves variations of the two middle β -strands and the less conserved loop of variable length that separates them. Some cupins exemplified by HAD, germin (oxalate oxidase) and germin-like proteins involved in the response of plants to pathogens and stresses are composed of a single domain. Other cupins are composed of two copies of the domain that probably arose from gene duplication. The group of two-domain cupins is exemplified by GO, 1-hydroxy-2-naphthoate dioxygenase, oxalate decarboxylase and seed storage proteins. A special class of cupins exemplified by AraC/XylS-type transcription factors and some helix–turn–helix transcription factors are composed of a cupin domain linked to a DNA binding domain. In these proteins, the cupin domain binds the effector molecule^{38, 39}.

In those cupins that possess a catalytic function, a metal ion is often present at the active site. When present, the metal ion ligands are found in each of the two β -sheet motifs. Cupins utilize as diverse a range of metal ions as the vicinal oxygen chelate superfamily: iron in GO⁵⁸, 1-hydroxy-2-naphthoate dioxygenase⁷⁰, HGO¹³⁸, HAD¹⁴⁸ and cysteine dioxygenase¹⁶¹; manganese in oxalate decarboxylase, germin (oxalate oxidase) and germin-like proteins^{3, 159}; zinc in phosphomannose isomerase²⁵; copper in quercetin 2,3-dioxygenase⁴⁶; nickel in acireductone dioxygenase¹¹¹ and nickel or cobalt in enolase-type enzymes³².

4. STRUCTURAL ASPECTS OF EXTRADIOL DIOXYGENASES

Crystal structures of Fe(II)-dependent extradiol and extradiol-type dioxygenases are now available in the ferrous (active) form for DHBD_{LB400} (Figure 4A)⁵⁴, DHBD from *Pseudomonas* sp. KKS102 (DHBD_{KKS102})¹⁴², C23O_{mt2}⁷⁶ and in the ferric (inactive) form for DHBD_{KKS102}¹²⁶, 4,5-PCD_{SYK-6} (Figure 4C)¹³⁵ and human HGO (Figure 4B)¹³⁸. Even though these enzymes represent each of the three families of extradiol and extradiol-type dioxygenases and possess the overall structural folds discussed in Section 3, they all share similar active sites and all have the same iron ligands, two histidines and one glutamate, that constitute the 2-His 1-carboxylate structural motif. Several other conserved residues identified through sequence alignments of each type of dioxygenases were observed at their respective active sites^{54, 135, 138}. Further investigation is required to probe the respective roles of these residues in the catalytic mechanism. This section focuses on those structural aspects of extradiol dioxygenases that are not discussed within the context of the classification (Section 3) or catalytic mechanism (Section 6) of these enzymes.

4.1. Type I Extradiol Dioxygenases

All Type I extradiol dioxygenases identified to date consist of a single type of subunit. The size of this subunit is typically 21 and 32.5 kDa for one- and two-domain enzymes, respectively. The vast majority of type I enzymes identified to date are two-domain, suggesting that the catalytically inactive N-domain confers some sort of advantage to the host. Steady-state kinetic characterization of one- and two-domain DHBDs in *R. globerulus* P6 suggests that the latter are significantly more catalytically efficient¹⁴⁵. It is thus tempting to speculate that the N-domain enables the C-domain to explore a greater range of sequence space, perhaps by conferring additional stability, thereby permitting the evolution of a more efficient active site. However, characterization of additional enzymes is necessary, particularly as the preferred substrates of the one-domain enzymes may not yet have been identified.

The one-domain enzymes are typically hexameric. In contrast, the two-domain enzymes exist in a range of oligomeric states. For example, 2,2',3-trihydroxybiphenyl 1,2-dioxygenase from *Sphingomonas* sp. RW1 is monomeric⁵⁵, C23O_{mt2} is tetrameric⁷⁶ and DHBD_{LB400} is octameric⁵⁴. The physiological significance of these different oligomeric states is not clear.

Sequence alignments⁴¹ and the structures of three different enzymes^{54, 76, 126} indicate that the tertiary structures of two-domain type I extradiol dioxygenases are very similar even though the sequence identities of

these enzymes can be less than 15%. The crystal structure of substrate-free DHBD_{LB400}, determined at a resolution of 1.9 Å⁵⁴, revealed that each monomer comprises one chain of 297 residues, the N-terminal methionine being excised. Each monomer possesses two domains of very similar structure (Figure 4A), the ferrous iron located in the C-terminal domain (C-domain). Each domain is made-up of two βαβββ modules as described in Section 3.1: modules 1 and 2 comprise the N-domain, modules 3 and 4, the C-domain. A large, funnel-shaped space lies entirely within the domain where the active site ferrous iron is ligated deep within this space in the C-domain. The C-domain possesses two additional β-strands after the common core structure and the central funnel is slightly larger than that of the N-domain. Therefore, evolutionary adaptation of the two-domain enzymes seems to have resulted in the loss of a second active site within the N-domain.

The ferrous iron active site is located midway in the 20 Å long funnel of the C-terminal domain. This funnel is opened at both ends, the large opening is 10 Å wide and the smaller opening is 6 Å wide. Thus the iron is probably only accessible to catecholic substrates from the wide opening, but water or O₂ can access the iron through either end. The coordination geometry of the iron is that of a well-defined square pyramid, with His146 as the axial ligand, and His210, Glu260 and two waters as equatorial ligands in the basal plane (Figure 5)⁵⁴. Spectroscopic studies of C23O_{mt2}^{87, 128} and DHBD_{LB400}³⁵ provide further evidence for the five-coordinate, square pyramidal geometry of the iron in the substrate-free enzyme.

The crystal structures of DHBD_{KKS102} with DHB^{124, 142} and DHBD_{LB400} with various catecholic substrates^{31, 143, 144} show that the latter bind to the iron inside the funnel-shaped cavity. The catechol ring binds in a restricted pocket that is highly complementary in size and shape. It is generally assumed that the crystallographically observed binding mode, which is similar in all reported complexes, is the one that leads to productive catalysis. One hydroxyl group of the substrate binds in the site trans to His146, whereas the other binds trans to His210, displacing the two ordered water ligands (Figure 6). Interestingly, a hydrogen bonded water bridges Asp244 and the 3-hydroxyl group. In some of the ES complexes, a water is also observed between His195 and the Fe. However, the degree of occupancy of this water site seems to depend on the crystal preparation¹⁴³. Indeed, it was not occupied in a recent structure of the DHBD_{KKS102}:DHB complex¹²⁴. Moreover, spectroscopic studies indicate that the C23O_{mt2}:catechol^{87, 128} and DHBD_{LB400}:DHB³⁵ complexes are 5-coordinate. This site is occupied by NO in the DHBD_{KKS102}:DHB:NO ternary complex¹²⁴ and likely represents the site for O₂ binding.

Sequence alignments reveal that the one-domain enzymes are similar in structure to the C-domain of type I extradiol dioxygenases. The one-domain enzymes are nevertheless approximately 65 residues larger than the typical

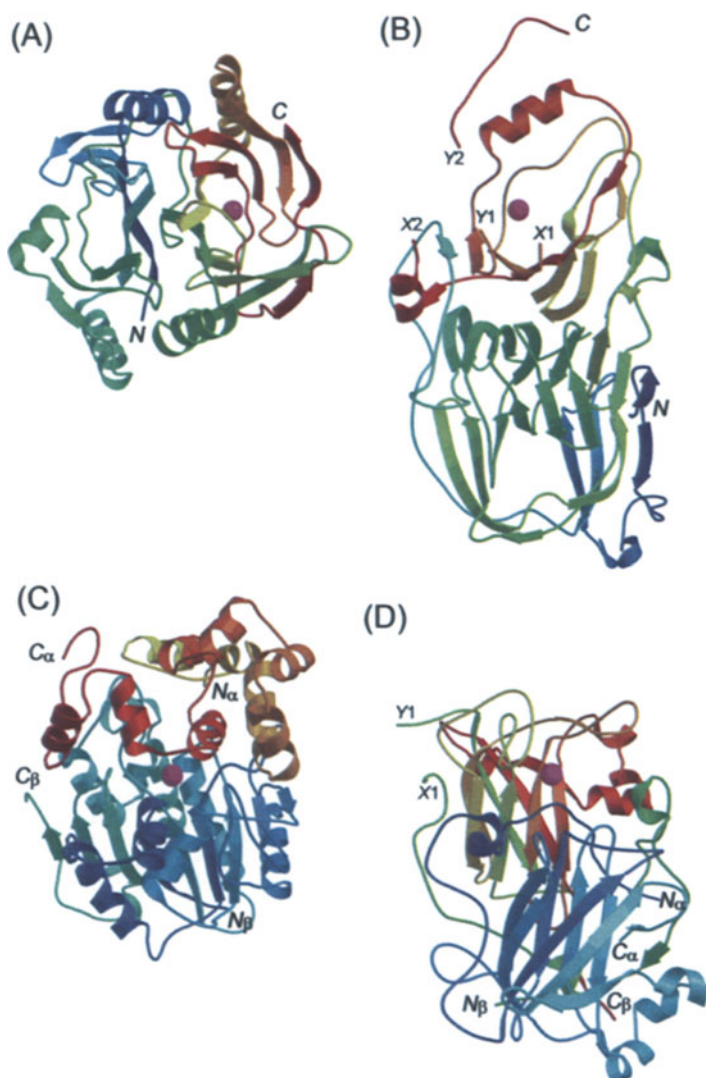


Figure 4. Ribbon drawings of the monomers of (A) DHBD_{LB400} and (B) HGO, and the protomers of (C) 4,5-PCD_{SYK-6} and (D) 3,4-PCD_{B-10}. Each drawing is on the same scale and the Fe atoms, which are drawn as magenta spheres, have been aligned at the same vertical position. For DHBD_{LB400} and HGO the course of the polypeptide backbone is color-ramped from blue (N-terminus) to red (C-terminus). For (C) 4,5-PCD_{SYK-6}, the larger β subunit is colored from blue (N-terminus) to blue-green (C-terminus), and the β chain is colored from yellow (N-terminus) to red (C-terminus). For 3,4-PCD_{B-10}, the larger α chain is colored from blue (N-terminus) to blue-green (C-terminus), and the β chain is colored from green (N-terminus) to red (C-terminus). The N- and C-terminal residues of each chain are labeled with *N* and *C*. In the drawings of HGO and 3,4-PCD_{B-10}, residues not resolved in the crystal structures are represented by breaks in the ribbons; symbols (*X1*, *Y1*) and (*X2*, *Y2*) mark the beginning (*X*) and end (*Y*) of breaks. Programs MOLSCRIPT⁸¹ and RASTER3D⁹⁴ were used to prepare the drawings.

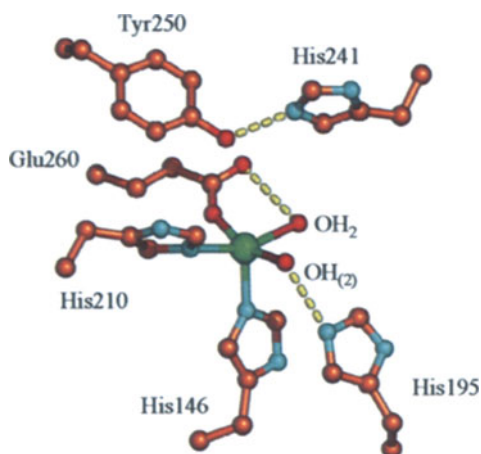


Figure 5. Structure of the active site of DHBD_{LB400}. Carbon, oxygen, nitrogen, and iron atoms are colored orange, red, cyan, and green, respectively. Hydrogen bonds are indicated by yellow dotted lines (adapted from ref. [54]; figure made using PyMol³⁶).

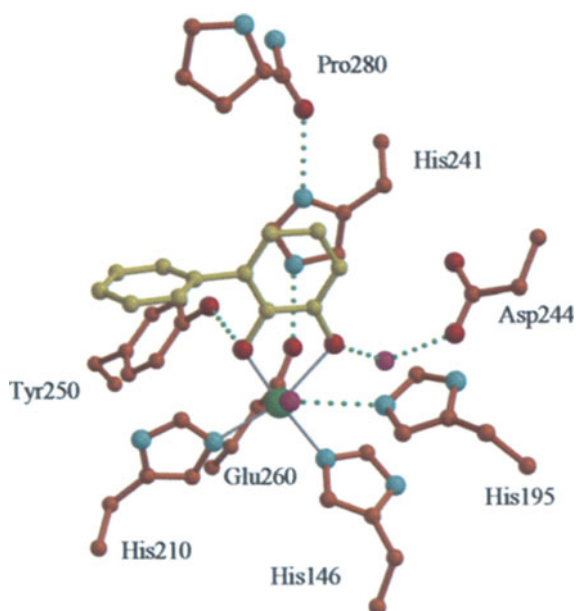


Figure 6. Hydrogen bonding in the active site of the DHBD_{LB400}:DHB complex. Carbon atoms are colored orange in protein residues and yellow in DHB. Oxygen, nitrogen, and iron atoms are colored red, cyan, and green, respectively. Fe-ligand bonds are indicated by gray sticks, and hydrogen bonds are indicated by green dotted lines (From ref. [143]. Reprinted with permission from the American Chemical Society.).

C-domain of two-domain enzymes. The structure of these residues is unknown, but they are presumably required to stabilize the catalytic domain of these enzymes.

Sequence alignments further suggest that no type I extradiol dioxygenases possesses an active site in the N-terminal domain⁴¹. However, an interesting module-swapping event may have occurred in the evolution of HQOs. More specifically, the first iron ligand in type I dioxygenases is a conserved histidine that is normally positioned at the beginning of the first β -strand of module 3 (His146 in DHBD_{LB400}). This residue is not conserved in HQOs. However, a histidine residue is conserved at a similar position at the beginning of the first β -strand of module 1. It is tempting to speculate that the two domains of the HQO subfamily of type I enzymes comprise modules 1 and 4 and modules 2 and 3, respectively, and that the former contains the active site.

4.2. Type II Extradiol Dioxygenases

As noted in Section 3, the characterized type II extradiol dioxygenases are all multimers possessing one or two different subunit types. For example, 2,3-dihydroxyphenylpropionate 1,2-dioxygenase¹³⁰, HPCD_{EcoC}¹²² and C23O_{JMP222-I}⁷³ are homooligomers, whereas 4,5-PCD_{SYK6}¹³⁵ and APD^{34, 136} have $\alpha_2\beta_2$ composition. In the case of 4,5-PCD_{SYK6}, the subunits appear to be unrelated, and the β subunit is similar to the protomers of the homooligomeric enzymes. In contrast, the two subunits of APD share sequence similarity, but it appears that only the β subunit contains an active site.

Of the type II enzymes, the only known structures present the ferric form 4,5-PCD_{SYK6} as the free-enzyme and in a binary complex with protocatechuate (Figure 4C)¹³⁵. The larger β subunit has 302 amino acids that form a globular α/β structure composed of 11 β strands, nine α helices and one 3_{10} helix. The 139 residues of the α subunit form 10 α -helices, which assemble into a rather noncompact plate-like structure that interacts extensively with one face of the β subunit of the same protomer and with the β subunit of the second protomer. The latter α - β contacts stabilize the $\alpha_2\beta_2$ dimer, which lacks α - α or β - β contacts.

The active site is located in a cleft in the β subunit on a surface that is extensively covered by the α subunit. The catalytically essential Fe is thus buried and is approximately 15 Å from the surface of the enzyme. In the substrate-free form of the enzyme, the Fe is coordinated in a distorted trigonal pyramidal geometry by His residues β 12 and β 61, Glu β 242 and one water molecule. The protein ligands form the base of the pyramid and the Fe is displaced from the basal plane toward the water ligand. A potential weak fifth ligand, Asn β 59, is located trans to the water at a distance of 2.9 Å. Although the protein ligands are identical in character to those observed for the type I

enzymes, the three-dimensional arrangement of the ligands is effectively enantiomeric in that one His and the Glu ligand exchange locations relative to the positions in DHBD_{LB400} and C23O_{mt2}. Binding of protocatechuate involves both hydroxyl groups and displaces the water ligand. The complex has a distorted trigonal bipyramidal geometry, with His β 61 and the 3-hydroxyl moiety as axial ligands.

4.3. The 2-His-1-Carboxylate Structural Motif

Despite the phylogenetic diversity of extradiol-type dioxygenases, they all contain a 2-His-1-carboxylate structural motif^{61, 117}. This structural motif is found in a wide variety of unrelated non-heme Fe(II) enzymes, including other microbial catabolic enzymes, illustrating its ability to provide a catalytic basis for diverse reactions. The active site of these enzymes contains an Fe(II) ligated by two histidines and one carboxylate all located on one face of the Fe(II) coordination sphere. This motif therefore provides up to three sites to ligate solvent species or substrate molecules on the other side of the coordination sphere. In addition to the three families of extradiol-type dioxygenases mentioned above, at least three additional families of enzymes have been identified to utilize this metal ion site: ring-hydroxylating dioxygenases, pterin-dependent hydroxylases, and α -ketoglutarate dependent enzymes. The latter include some closely related enzymes that do not utilize α -ketoglutarate as a cosubstrate. Ring-hydroxylating dioxygenases, exemplified by naphthalene and biphenyl dioxygenases^{69, 74}, catalyze the NADH-mediated *cis*-dihydroxylation of an arene double bond yielding a *cis*-diol. The Fe(II) of these enzymes has two available sites as it is ligated by the two oxygens of the carboxylate group. Pterin-dependent hydroxylases, such as mammalian phenylalanine and tyrosine hydroxylases⁴⁴, use tetrahydrobiopterin as a cofactor to hydroxylate the ring of aromatic amino acids residue in the synthesis of brain signaling molecules. A homolog in *Pseudomonas aeruginosa* hydroxylates phenylalanine to tyrosine¹⁶⁵. Examples of α -ketoglutarate-dependent enzymes and related enzymes include enzymes involved in the synthesis of β -lactam antibiotics. Among these enzymes, isopenicillin N synthase¹²⁰ requires no cofactor, whereas deacetoxycephalosporin C synthase¹⁴⁷ and clavaminic acid synthase¹⁶⁴ both require α -ketoglutarate as a cosubstrate to facilitate their respective reactions. Another member of this family that does not require α -ketoglutarate as a co-substrate is 1-aminocyclopropane-1-carboxylic acid oxidase⁵³, an enzyme involved in the formation of the plant signaling molecule ethylene. This enzyme requires ascorbate as a cofactor and CO₂ as an activator for continuous turnover. Interestingly, the enzymes in this family have recently been suggested to be members of the cupin superfamily based on their similar distorted jelly roll β -barrel structure⁶³.

The remarkable range of reactions catalyzed by this type of Fe(II) center can be explained by the mechanism of these enzymes that always involves binding of oxygen atoms to the open sites of the Fe(II) center. This motif can be seen as the counterpart of the heme cofactor where only one site is available for endogenous ligand compared to three for this motif. The close proximity of the three open sites allows the juxtaposition of the two reactants to promote catalysis. In addition to the enzymes described here, iron superoxide dismutase⁸⁴ and lipoxygenase⁹⁵ also have this structural motif. However, they have an additional histidine ligand, which alters the role of the iron as it shuttles between the Fe(III) and Fe(II) oxidation states during catalysis. The contrast between these two enzymes and the Fe(II) oxygen activating enzymes shows the flexibility of the 2-His 1-carboxylate motif.

5. STRUCTURAL ASPECTS OF INTRADIOL DIOXYGENASES

More than 20 crystal structures representing three distinct intradiol dioxygenases are currently available in the Protein Data Bank. The type member of the class is 3,4-PCD_{B-10}, which is a large, dodecameric assembly with tetrahedral symmetry^{103, 104, 105}. The protomer comprises two chains of related structure, α and β , and binds one ferric Fe atom at the active site (Figure 4D). The oligomer resembles a porous and hollow, truncated tetrahedron with an edge length of approximately 180 Å. Contacts between protomers are largely mediated by the β subunits, which interact extensively across tetrahedral 2-fold axes to form an inner shell surrounding a central cavity of approximately 50 Å in diameter. The α subunits associate around the 3-fold axes at each apex and coincidentally lie at the corners of the opposing bases, which have a central pore outlined by β subunits. The crystal structure of a homolog from *Acinetobacter* sp. strain ADP1, 3,4-PCD_{ADP1}, demonstrates the same ($\alpha\beta\text{Fe(III)}$)₁₂ quaternary structure¹⁵⁰, whereas other 3,4-PCDs utilize the same protomer in a variety of oligomeric states¹⁰³.

The α and β subunits of 3,4-PCDs comprise approximately 200 and 230 residues, respectively, and are homologous but divergent^{103, 104}. The level of identity between α and β subunits is 30% for 3,4-PCD_{B-10} and 26% for 3,4-PCD_{ADP1}, whereas alignments between the two species yield 49% and 56% for the α and β subunits, respectively¹⁵⁰. The secondary structure of the subunits is nearly all β and is dominated by a β sandwich described as an eight-stranded sheet folded in half to form two layers. The β sandwich and two large connecting loops form a structurally conserved core such that 127 equivalent C α atoms from the two chains of 3,4-PCD_{B-10} can be superimposed with an rms deviation of 1.04 Å¹⁰⁵. Although the β sandwich is a reasonably common

structural motif, the topology of the core structure is so far unique to the intradiol dioxygenases¹⁰³.

The active site of each protomer is located at one end of the extensive interface between the α and β subunits near a 3-fold apex of the oligomer and is accessible from outside the protein. The β chain provides most of the residues in the vicinity of the Fe, although a short segment from near the N-terminus of the α chain (3–4 residues near residue 15) completes the active site. In the substrate-free enzyme, the Fe is bound by one water ligand and four protein side chain ligands supplied by the β subunit, including two tyrosines and two histidines. The ferric iron of substrate-free intradiol dioxygenase has a distorted trigonal bipyramid geometry, with a tyrosine, a histidine and a solvent species coordinated in the equatorial plane and a tyrosine and a histidine coordinated in the axial positions (Figure 7A)¹⁰⁵. The structures of a variety of binary complexes with substrates and substrate analogs indicate displacement of the axial tyrosine accompanies formation of productive complexes¹⁰⁸. The structures of a series of competitive inhibitors suggest substrate binding may involve several stages prior to formation of the reactive complex¹⁰⁷.

The recent crystal structure of C12O_{ADPI} defined a second structural class of intradiol dioxygenases¹⁴⁹. This enzyme is a homodimer of a 311 residue subunit. The subunit includes a catalytic domain that replicates the

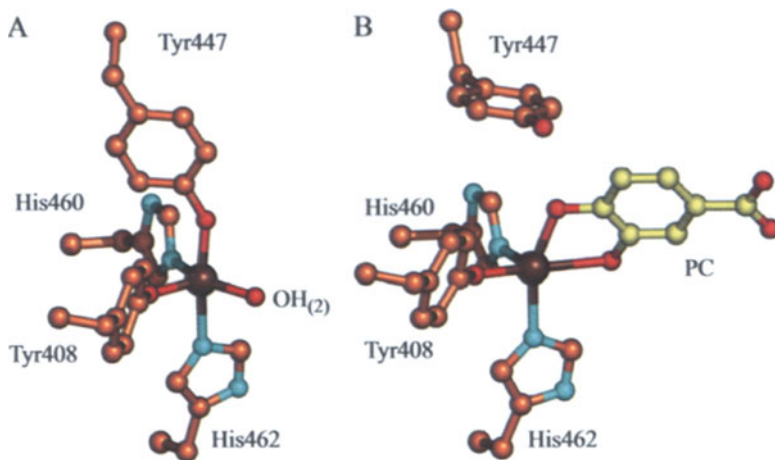


Figure 7. Structure of the active site of (A) substrate-free and (B) protocatechuate-bound (B) 3,4-PCD_{B-10}. Carbon atoms are colored orange in amino acids and yellow in protocatechuate. Oxygen, nitrogen, and iron atoms are colored red, cyan, and brown, respectively (adapted from refs [105] and [108]; figure made using PyMol³⁶).

basic core structure of the 3,4-PCDs joined to an N-terminal, all-helical dimerization domain, which includes five helices and approximately 100 residues. The core structure provides four protein ligands in locations equivalent to those provided by the β subunit in the 3,4-PCDs. An extended segment that links the dimerization domain to the catalytic domain replaces the short active site segment of the 3,4-PCD α chain. The coordination of the Fe in the substrate-free enzyme is largely the same, and substrate binding displaces the axial tyrosine.

6. MECHANISM OF EXTRADIOL DIOXYGENASES

The catalytic strategy utilized by the different types of extradiol dioxygenases appears to be similar and the proposed mechanism is based on studies of members of each family¹⁶. In the first step of this mechanism (Figure 8), the catecholic substrate binds to the ferrous iron in a bidentate manner, displacing the two solvent ligands^{4, 5, 87, 142, 143, 144}. Spectroscopic data demonstrate that DHBD_{LB400} binds its preferred substrate, DHB, as a monoanion¹⁴³ as had been inferred from XAS studies of C23O_{mt2}¹²⁸. The observed asymmetric binding of DHB to DHBD_{LB400} ($r_{\text{Fe-O}} = 2.0$ and 2.4 \AA)¹⁴³ indicates that O-2

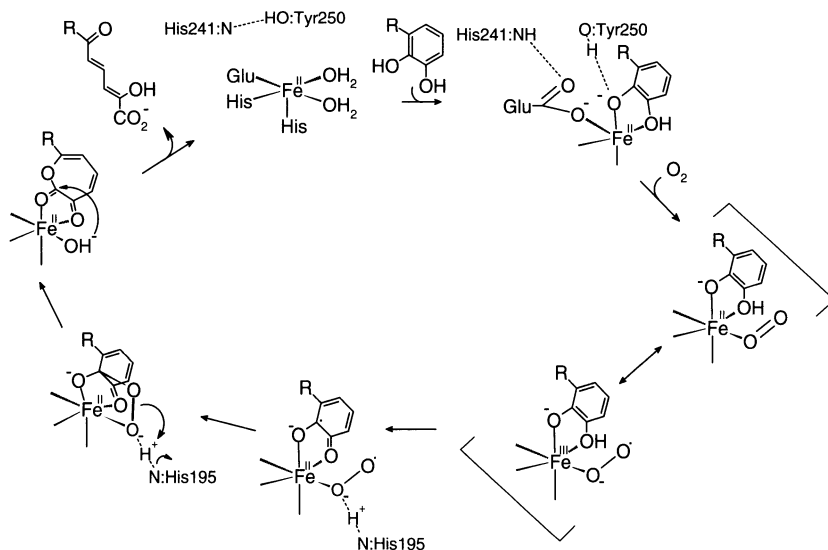


Figure 8. Proposed mechanism of extradiol dioxygenases with the role of conserved active site residues (adapted from refs [16] and [128]). For clarity, the displacement of solvent species from the ferrous center is not depicted explicitly.

is deprotonated, but not O-3. The binding of the catecholic substrate to the iron activates the latter for O₂ binding^{5, 87, 128}. Subsequent steps in the catalytic mechanism are less well substantiated. Biochemical studies provide some support for a mechanism involving iron-mediated transfer of an electron from the catechol to the O₂, yielding a semiquinone-Fe(II)-superoxide intermediate¹³¹. This species is proposed to react to give an iron-alkylperoxo intermediate¹⁵⁷, which undergoes alkenyl migration, Criegee rearrangement and O–O bond cleavage to give an unsaturated lactone intermediate and an Fe(II)-bound hydroxide ion. The latter hydrolyzes the lactone to yield the reaction product¹²³.

The crystal structures of various complexes of DHBD_{LB400} and DHBD-KKS102^{124, 142, 143, 144} suggest roles for the different conserved active site residues in the mechanism of extradiol dioxygenases. The structural data strongly suggest that the conserved active site His241 of DHBD_{LB400} assists in the deprotonation of the catechol in the enzyme-catalyzed reaction (Figure 9). In the substrate-free enzyme, His241 is presumed to be in the imidazole (neutral) state because of its proximity to the Fe(II) atom and its hydrogen bonding interactions. The substrate-induced structural changes (Figure 9)¹⁴³ observed for His241 are consistent with its protonation. In the enzyme:DHB complex, the side chain of His241 is stacked with the catechol ring and His241 and Glu260 have rotated toward each other forming a new hydrogen bond. This change implies that His241 has acquired a proton during formation of the ES complex. Tyr250 forms a new hydrogen bond with the 2-hydroxyl oxygen of the substrate. Tyr250 could act as a proton shuttle between the catecholic substrate and His241.

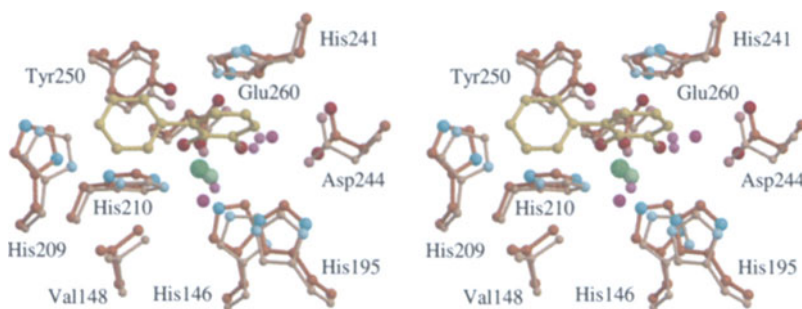


Figure 9. Displacements of active site atoms/residues associated with DHB binding in DHBD_{LB400}. This is a (divergent) stereoscopic diagram. Atoms and bonds in the substrate-free structure are represented by smaller spheres, thinner sticks and lighter shades. Carbon atoms are colored orange in protein residues and yellow in DHB. Nitrogen and iron atoms are cyan and green, respectively. Protein and DHB oxygen atoms are red, whereas water oxygens are magenta (From ref. [143]. Reprinted with permission from the American Chemical Society.).

In the subsequent step of the proposed mechanism, O₂ binds to the ferrous iron. As shown in Figure 8, the dominant form of this species would be Fe(III)-O₂⁻. Formation of Fe(III) would induce deprotonation of the 3-hydroxyl, forming the dianion chelate, as proposed in extradiol enzymes^{40, 108, 150}. The proton could be picked up by the iron-bound superoxide. Interestingly, Ne2 of the conserved His195 is positioned within 3 Å of the proximal O atom in the modeled DHBD_{LB400}:DHB:O₂ ternary complex¹⁵, and could thus stabilize protonation of that particular O atom of the superoxide species. A similar role for this histidine in deprotonating the 3-hydroxyl group of the substrate and then stabilizing the superoxide species was suggested based on the crystal structure of a DHBD_{KKS102}:DHB:NO ternary complex¹²⁴. Electron transfer from the bound catechol would produce an Fe(II)-semiquinone. Attack of the activated oxygen species in a pseudo-axial position at C-2 satisfies the orbital steering requirements proposed by Bugg to be critical for extradiol cleavage¹⁶. In the subsequent step, the proton originating from the 3-hydroxyl group would assist in the heterolysis of the O-O bond in the proposed Criegee rearrangement that results in the lactone and the Fe-hydroxide. Finally, hydrolysis of the lactone by the Fe-bound hydroxide, and release of the proton on His241 complete the catalytic cycle. This mechanism (Figure 8) does not rule out alternate roles for His195 and His241. However, it illustrates the importance of the Fe(II)-bound monoanion in coordinating electron and proton transfer upon O₂ binding.

7. INACTIVATION OF EXTRADIOL DIOXYGENASES

Kinetic analyses have established that extradiol dioxygenases are subject to two forms of substrate inhibition, reversible substrate inhibition and a mechanism-based inactivation (or suicide inhibition), as well as oxidative inactivation in the absence of substrate. As discussed in greater detail in Sections 7.1 and 7.2, inactivation in the absence and presence of substrate are quite similar as both involve oxidation of the active site iron.

Reversible substrate inhibition has been reported in a number of enzymes including DHBD_{LB400}^{42, 144}, DHBD_{CB15}¹, THBD_{RW1}⁵⁵, DHBD_{P6}⁹, DHBD_{BN6}⁶² and 2,3-dihydroxyphenyl-propionate dioxygenase¹³⁰. Interestingly, it has only rarely been reported for C23Os¹¹⁰. However, other than in DHBD_{LB400}, it is not clear what proportion of the decrease in the initial rate of DHB-cleavage at high concentrations of DHB is due to reversible substrate inhibition and irreversible suicide inhibition, respectively. Notably, the initial rates of cleavage of substituted catechols by DHBD_{BN6} could not be fitted to the substrate inhibition equation⁶². DHBD_{LB400} is clearly subject to both modes of inhibition by both DHB and 3-ethylcatechol¹⁴⁴. While the mechanism of reversible substrate

inhibition is not clear, it is unlikely to involve negative cooperativity between the subunits of DHBD_{LB400} as it has also been reported for a monomeric enzyme⁵⁵. It is possible that DHB could occupy the auxiliary *t*-butanol binding site observed in the DHBD_{LB400}:DHB complex¹⁴⁴, thereby inhibiting the cleavage reaction in a similar manner.

7.1. Mechanism-Based Inactivation

One physiologically significant aspect of extradiol dioxygenase function is mechanism-based inactivation. Although it was described over 20 years ago in C23O_{mt2}⁷⁸, the phenomenon was first recognized in mammalian HAD⁹⁶. It has since been studied in APD_{JS45}³⁴ and DHBD_{LB400} of the *bph* pathway¹⁴⁶. Mechanism-based inactivation has been proposed to limit the range of toluates metabolized by the TOL pathway²³ and the extent of transformation of PCBs by the *bph* pathway¹⁴⁶.

The molecular basis of the mechanism-based inactivation of extradiol dioxygenases has been subject to some debate. Thus, the inactivation of C23O_{mt2} by 3-chlorocatechol has been suggested to occur either through reversible chelation of the active site iron⁷⁸ or irreversible covalent modification by an acyl chloride species generated by the ring cleavage reaction¹¹. However, no evidence for either mechanism has been presented. In contrast, the inactivation of C23O_{mt2} by alkyl catechols appears to involve the accidental oxidation of the active site Fe(II) to Fe(III) during turnover²³. Interestingly, a halogenated substrate analog, 4-chloro-3-hydroxyanthranilate, had been suggested to inhibit HAD via covalent modification by an acyl halide¹⁰⁹, although it was subsequently shown that this analog inhibits the enzyme reversibly in vivo¹⁵¹.

Recent studies of DHBD_{LB400} indicate that the mechanism-based inactivation of this enzyme in the presence of a variety of catechols including 3-chlorocatechol and DHB involves the dissociation of superoxide from the EAO₂ ternary complex with the concomitant oxidation of the active site Fe(II) (Figure 10)¹⁴⁶. More particularly, in vitro studies demonstrated that this inactivation results in the formation of Fe(III) and was reversed by anaerobic incubation of the inactivated enzyme with Fe(II) and a reducing agent. Moreover, the mechanism-based inactivation of DHBD_{LB400} does not involve covalent modification, as judged by a lack of change to the molecular mass of DHBD_{LB400} inactivated by 3-chlorocatechol or other catechols. Further evidence for this conclusion comes from in vivo studies in which 3-chlorocatechol-inactivated DHBD_{LB400} was readily reactivated in the absence of protein synthesis. The dissociation of superoxide from the EAO₂ complex prior to formation of an iron-alkylperoxo intermediate is consistent with the proposed catalytic mechanism of extradiol dioxygenases and may represent a general means by which these enzymes are inactivated during catalytic turnover.

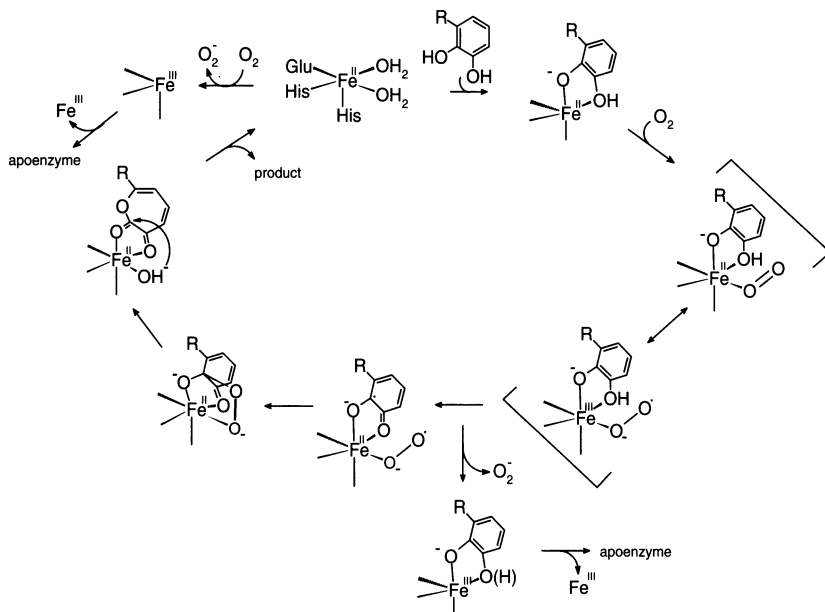


Figure 10. General mechanism of inactivation of extradiol dioxygenases. The exact step at which superoxide dissociates from the ternary complex has not been determined. The ligands in the ferric form of the enzyme are unknown (adapted from refs [16], [124], and [146]). For clarity, the displacement of solvent species from the ferrous center is not depicted explicitly.

Consistent with this suggestion, catechol-inactivated APD_{JS45} can be reactivated upon incubation with Fe(II) and a reducing agent³⁴. Moreover, one study reported that the inactivation of C23O_{mt2} by 3-chlorocatechol also involves oxidation of the active site Fe(II)¹⁵⁵. It should be noted that while 3-chlorocatechol inactivates many catechol-cleaving extradiol dioxygenase, CC23O_{GJ31}, which is related to C23O_{mt2}⁸⁹ catalyzes the efficient cleavage of 3-chlorocatechol⁸⁸. However, how this enzyme accomplishes this remains unclear.

For both DHBD_{LB400} and C23O_{mt2}, oxidative inactivation is more marked for poorer substrates^{22, 146}, suggesting that the substrate-binding pocket of these enzymes is tuned both to maximize specificity for a particular substrate and minimize inactivation during catalytic turnover. Interestingly, the TOL pathway contains a 2Fe–2S ferredoxin to reactivate C23O, thereby increasing the range of substrates that the organism utilizes as sole source of carbon and energy¹¹². The ferredoxin is encoded by *xyIT*, which is located immediately upstream of the C23O-encoding *xyIE*. Homologous ferredoxins are found in a number of catabolic pathways^{68, 139}. Interestingly, the partition ratio of C23O for catechol is 1,400,000²³, indicating that DHBD_{LB400} is much more susceptible than is C23O_{mt2} to suicide inactivation by its putative preferred substrate. Despite the higher susceptibility of DHBD_{LB400} to suicide

inactivation, no such ferredoxin has been associated with the *bph* pathway. However, the in vivo reactivation of 3-chlorocatechol-inactivated DHBD_{LB400} in *Burkholderia* sp. LB400 and *E. coli* suggests that a nonspecific electron transfer protein can play this role¹⁴⁶.

7.2. Oxidative Inactivation in the Absence of Substrate

Extradiol dioxygenases are also susceptible to oxidative inactivation in the absence of substrate. This process also apparently involves binding of O₂ to the active site iron and the loss of superoxide¹⁴⁶. It is possible that the high K_m value of many extradiol dioxygenases for O₂ (K_{mO_2}) reflects the low affinity of the free enzyme for O₂, which may have evolved as a protective adaptation against oxidative inactivation. For example, the K_{mO_2} of DHBD_{LB400} and 2-aminophenol dioxygenase are 1.3 mM¹⁴⁴ and 710 μ M⁸⁵, respectively. Interestingly, C23O_{mt2}, which is less susceptible to O₂-dependent inactivation¹⁰¹, has a lower K_{mO_2} (10 μ M^{66, 79}). Moreover, the growth rate of *P. putida* mt-2 on benzoate at different pO₂ is limited by the K_{mO_2} of C23O⁸. It is clear that some C23Os have evolved to function in microaerobic environments, and thus have even lower K_{mO_2} (0.7 μ M⁸²). In the latter case, there is presumably less O₂ to inactivate the C23O.

The oxidative inactivation of extradiol dioxygenases in the absence of substrate complicates their purification and characterization using aerobic buffers. This problem can be at least partially alleviated through the inclusion of organic additives such as isopropanol, *t*-butanol and acetone in solutions of the enzymes^{42, 79, 101, 144}. These additives were also used in crystallographic studies to stabilize the enzymes. The crystallographic data from DHBD_{LB400}^{54, 144} and C23O_{mt2}⁷⁶ indicate that these additives occupy the active site, close to the catalytic iron center thereby stabilizing the active site and/or protecting the iron from direct access by oxidants or substrates. Consistent with this notion, *t*-butanol competitively inhibited DHBD_{LB400}¹⁴⁴. Moreover, *t*-butanol and isopropanol, which inhibit DHBD_{LB400} more effectively than ethanol and glycerol, also stabilize the enzyme more effectively. Interestingly, acetone competitively inhibits C23O_{mt2} more effectively than *t*-butanol inhibited DHBD_{LB400}^{79, 101}. This suggests that C23O_{mt2} has a much higher affinity for acetone than DHBD_{LB400} has for *t*-butanol, and may explain why the former is so much more stable in acetone-containing buffers⁷⁹ than is DHBD_{LB400} in *t*-butanol-containing buffers. In C23O_{mt2}, it was even proposed that acetone binds directly to the iron^{14, 76}. However, this direct binding remains to be clarified as the precision of the respective experiments was limited. Inspection of the *t*-butanol binding site in DHBD_{LB400} and of the acetone binding site in C23O reveals that it is partly formed by non-conserved residues, suggesting that the best organic stabilizer, if any, will be isozyme-specific.

Even with the addition of organic additives, the best reported aerobic preparations of DHBD_{LB400} contain at most 50% of their complement of active site Fe(II)⁴². This variability in preparations of extradiol dioxygenases complicates spectroscopic studies and the determination of steady-state kinetic parameters. For example, the k_{cat} of C23O_{mt2} has been variously reported as 930 s⁻¹ (100 mM phosphate, pH 7.5, 25°C;²²) and 278 s⁻¹ (50 mM phosphate, pH 7.5, 25°C;⁷⁹). These results demonstrate the value of anaerobic purification, and further illustrate the importance of calculating steady-state parameters as a function of the metal content of enzyme preparations¹⁴⁴.

8. MECHANISM OF INTRADIOL DIOXYGENASES

The proposed mechanism of intradiol dioxygenases has been developed based on biochemical, spectroscopic and structural studies of 3,4-PCDs and C12Os (Figure 11)¹⁶. As in extradiol dioxygenases, the intradiol enzymes utilize an ordered mechanism in which catechol binding precedes O₂ reactivity^{18, 66, 152}. However, whereas extradiol enzymes activate the O₂ for nucleophilic attack on the catechols, intradiol enzymes appear to activate the catechols for electrophilic attack by O₂. In intradiol dioxygenases, catechol binding is a multi-step process that ultimately results in displacement of an axial tyrosine and an equatorial hydroxide ion to yield a bidentate bound catecholate (Figure 7B)^{45, 108, 140, 149, 150}. It is generally accepted that the displaced tyrosyl and hydroxide ligands accept the two hydroxyl protons of the substrate such

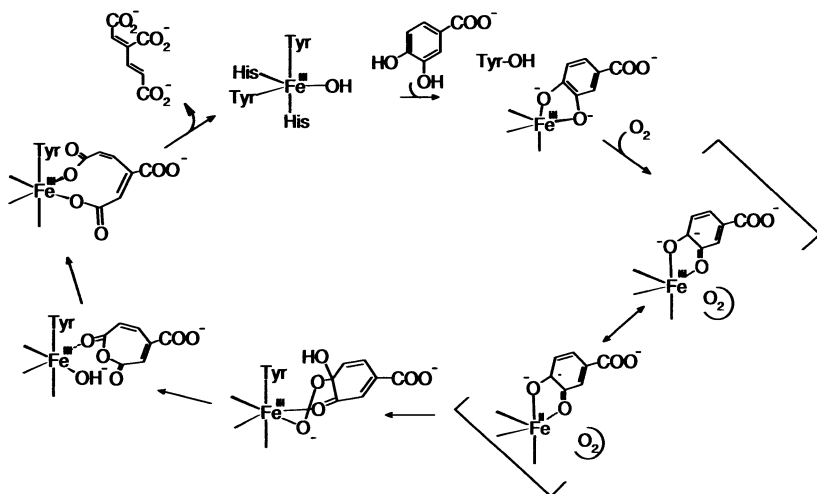


Figure 11. Reaction mechanism proposed for 3,4-PCD (adapted from refs [16] and [108]).

that the latter binds as a dianion¹⁰⁷. In the next step, O₂, thought to be sequestered in a hydrophobic pocket, attacks the bound catecholate directly, before coordinating to the iron and yielding an iron-alkylperoxo intermediate¹¹⁸. Although recent evidence indicates that this intermediate is similar in structure to that of the extradiol reaction¹⁵⁷, in the case of intradiol enzymes, the Criegee rearrangement and O–O bond cleavage involve acyl migration to yield the cyclic anhydride and an iron-bound oxide or hydroxide. The latter functions as a nucleophile to hydrolyze the anhydride and yield the ring-opened product.

The protonation state of the substrate is largely based on crystallographically determined bond lengths, which indicate that the substrate is asymmetrically bound: the long Fe–O bond is *trans* to a tyrosinate ligand and the short Fe–O bond is *trans* to a neutral histidine ligand^{108, 149, 150}. The asymmetry is proposed to reflect ketonization of the bond *trans* to the tyrosine. A survey of the structures in the PDB database (3PCA, 1EOB, 1DLT) reveals that the Fe–O bond lengths are similar to those observed in ES complexes of extradiol enzymes ($r_{\text{Fe-O}} = 2.0 \pm 0.1 \text{ \AA}$ and $2.4 \pm 0.2 \text{ \AA}$). Visible resonance Raman studies using the ligand-to-metal ion charge transfer bands show that 4-nitrocatechol and 3,4-dihydroxyphenylacetate bind to 3,4-PCD as dianions^{40, 114}. UV/Vis absorption spectroscopy corroborates dianionic binding of 4-nitrocatechol to 3,4-PCD and C12O^{141, 150}. 4-Nitrocatechol is an inhibitor of 3,4-PCD and 3,4-dihydroxyphenylacetate is a very poor substrate. Thus, these analogs may not bind in the same manner as the preferred substrate of the enzyme, PCA. However, structural data indicate that 3,4-PCD binds 3,4-dihydroxyphenylacetate and PCA in a similar manner. Considering the proposed importance of dianionic binding of the substrate to substrate activation in the catalytic mechanism of intradiol enzymes¹⁰⁸, it would be useful to obtain direct evidence for the protonation state of the bound substrate. This can probably be achieved using UVRRS¹⁰.

9. MECHANISTIC COMPARISON OF EXTRADIOL AND INTRADIOL DIOXYGENASES

The stereo-electronic factors that determine extradiol versus intradiol cleavage from the common intermediate have been proposed to involve the orientation of the iron-alkylperoxo moiety relative to the organic substrate. In particular, the extradiol dioxygenases are proposed to form a pseudo-axial iron-alkylperoxo species that would favor alkenyl migration and the intradiol dioxygenases are proposed to form a pseudo-equatorial iron-alkylperoxo species that would favor acyl migration¹⁶. The hypothesis that extradiol cleavage involves alkenyl migration whereas intradiol cleavage involves acyl

migration is supported by the range of compounds that are known substrates for these enzymes. Assuming that intradiol and extradiol ring cleavage proceed via a similar iron alkylperoxo intermediate, acyl migration can only occur in the known substrates of intradiol dioxygenases: catechols and substituted derivatives thereof. In contrast, the cleavage of compounds such as gentisate, which do not have vicinal hydroxyl groups, can only proceed via alkenyl migration, consistent with the extradiol dioxygenase mechanism.

An important difference in the initial stages of the proposed extradiol and intradiol mechanisms is the protonation state of the bidentate-bound catechol in the enzyme:substrate complex¹¹⁵. Thus, in extradiol dioxygenases, a monoanionic Fe(II)-bound catecholate activates the ferrous center for O₂-binding^{86, 128, 143}. By contrast, in intradiol dioxygenases, a dianionic Fe(III)-bound catecholate promotes direct electrophilic attack of the substrate by O₂, a reaction that is further favored by ketonization of the catecholate¹¹⁸. Despite the proposed significance of the different protonation states of the substrate in the two enzymes, the evidence for dianionic binding in the intradiol dioxygenase is not definitive. Thus, structural^{140, 108, 142, 143, 150} and EXAFS^{128, 154} data demonstrate that in both enzymes, the substrate is asymmetrically bound: one Fe-O bond is shorter than the other. In the case of intradiol dioxygenases, this has usually been interpreted as a ketonized dianion^{40, 108, 150, 154}. Most of the electronic absorption and resonance Raman data supporting this interpretation were obtained using poor substrates or inhibitors^{40, 114, 141, 150} which may not bind in the same manner as preferred substrates. Direct evidence for the protonation state of a preferred substrate bound to an intradiol dioxygenase could presumably be obtained using the same approach that has been used to study an extradiol dioxygenase¹⁴³.

REFERENCES

1. Adams, R.H., Huang, C.-M., Higson, F.K., Brenner, V., and Focht, D.D., 1992, Construction of a 3-chlorobiphenyl-utilizing recombinant from an intergeneric mating. *Appl. Environ. Microbiol.*, 58:647–654.
2. Alder, E., 1977, Lignin chemistry—past, present and future. *Wood Sci. Technol.*, 11:169–218.
3. Anand, R., Dorrestein, P.C., Kinsland, C., Begley, T.P., and Ealick, S.E., 2002, Structure of oxalate decarboxylase from *Bacillus subtilis* at 1.75 Å resolution. *Biochemistry*, 41:7659–7669.
4. Arciero, D.M. and Lipscomb, J.D., 1986, Binding of ¹⁷O-labeled substrate and inhibitors to protocatechuate 4,5-dioxygenase-nitrosyl complex. Evidence for direct substrate binding to the active site Fe²⁺ of extradiol dioxygenases. *J. Biol. Chem.*, 261:2170–2178.
5. Arciero, D.M., Orville, A.M., and Lipscomb, J.D., 1985, [¹⁷O]Water and nitric oxide binding by protocatechuate 4,5-dioxygenase and catechol 2,3-dioxygenase. Evidence for binding of exogenous ligands to the active site Fe²⁺ of extradiol dioxygenases. *J. Biol. Chem.*, 260:14035–14044.

6. Armengaud, J., Timmis, K.N., and Wittich, R.M., 1999, A functional 4-hydroxysalicylate/hydroxyquinol degradative pathway gene cluster is linked to the initial dibenzo-p-dioxin pathway genes in *Sphingomonas* sp. strain RW1. *J. Bacteriol.*, 181:3452–3461.
7. Armstrong, R.N., 2000, Mechanistic diversity in a metalloenzyme superfamily. *Biochemistry*, 39:13625–13632.
8. Arras, T., Schirawski, J., and Unden, G., 1998, Availability of O₂ as a substrate in the cytoplasm of bacteria under aerobic and microaerobic conditions. *J. Bacteriol.*, 180:2133–2136.
9. Asturias, J.A., Eltis, L.D., Prucha, M., and Timmis, K.N., 1994, Analysis of three 2,3-dihydroxybiphenyl 1,2-dioxygenases found in *Rhodococcus globerulus* P6. Identification of a new family of extradiol dioxygenases. *J. Biol. Chem.*, 269:7807–7815.
10. Barbosa, C.J., Vaillancourt, F.H., Eltis, L.D., Blades, M.W., and Turner, R.F.B., 2002, The power distribution advantage of fiber-optic coupled ultraviolet resonance Raman spectroscopy for bioanalytical and biomedical applications. *J. Raman Spectrosc.*, 33:503–510.
11. Bartels, I., Knackmuss, H.-J., and Reineke, W., 1984, Suicide inactivation of catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2 by 3-halocatechols. *Appl. Environ. Microbiol.*, 47:500–505.
12. Benvenuti, M., Briganti, F., Scozzafava, A., Golovleva, L., Travkin, V.M., and Mangani, S., 1999, Crystallization and preliminary crystallographic analysis of the hydroxyquinol 1,2-dioxygenase from *Nocardioides simplex* 3E: A novel dioxygenase involved in the biodegradation of polychlorinated aromatic compounds. *Acta Crystallogr. D Biol. Crystallogr.*, 55:901–903.
13. Bernat, B.A., Laughlin, L.T., and Armstrong, R.N., 1997, Fosfomycin resistance protein (FosA) is a manganese metalloglutathione transferase related to glyoxalase I and the extradiol dioxygenases. *Biochemistry*, 36:3050–3055.
14. Bertini, I., Briganti, F., and Scozzafava, A., 1994, Aliphatic and aromatic inhibitors binding to the active site of catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2. *FEBS Lett.*, 343:56–60.
15. Bolin, J.T. and Eltis, L.D., 2001, 2,3-Dihydroxybiphenyl 1,2-dioxygenase, In A. Messerschmidt, R. Huber, T. Poulos, and K. Wieghardt (eds), *Handbook of Metalloproteins*, pp. 632–642. John Wiley & Sons, Chichester, UK.
16. Bugg, T.D.H. and Lin, G., 2001, Solving the riddle of the intradiol and extradiol catechol dioxygenases: How do enzymes control hydroperoxide rearrangements? *Chem. Commun.*, 2001:941–952.
17. Bugg, T.D.H., 1993, Overproduction, purification and properties of 2,3-dihydroxyphenylpropionate 1,2-dioxygenase from *Escherichia coli*. *Biochim. Biophys. Acta*, 1202:258–264.
18. Bull, C., Ballou, D.P., and Otsuka, S., 1981, The reaction of oxygen with protocatechuate 3,4-dioxygenase from *Pseudomonas putida*. Characterization of a new oxygenated intermediate. *J. Biol. Chem.*, 256:12681–12686.
19. Cain, R.B., 1968, Anthranilic acid metabolism by microorganisms. Formation of 5-hydroxyanthranilate as an intermediate in anthranilate metabolism by *Nocardia opaca*. *Antonie Van Leeuwenhoek*, 34:17–32.
20. Cameron, A.D., Olin, B., Ridderstrom, M., Mannervik, B., and Jones, T.A., 1997, Crystal structure of human glyoxalase I—evidence for gene duplication and 3D domain swapping. *EMBO J.*, 16:3386–3395.
21. Catelani, D., Colombi, A., Sorlini, C., and Treccani, V., 1973, Metabolism of biphenyl. 2-Hydroxy-6-oxo-6-phenylhexa-2,4-dienoate: The meta-cleavage product from 2,3-dihydroxybiphenyl by *Pseudomonas putida*. *Biochem. J.*, 134:1063–1066.
22. Cerdan, P., Rekik, M., and Harayama, S., 1995, Substrate specificity differences between two catechol 2,3-dioxygenases encoded by the TOL and NAH plasmids from *Pseudomonas putida*. *Eur. J. Biochem.*, 229:113–118.

23. Cerdan, P., Wasserfallen, A., Rekik, M., Timmis, K.N., and Harayama, S., 1994, Substrate specificity of catechol 2,3-dioxygenase encoded by TOL plasmid pWWO of *Pseudomonas putida* and its relationship to cell growth. *J. Bacteriol.*, 176:6074–6081.
24. Chauhan, A., Samanta, S.K., and Jain, R.K., 2000, Degradation of 4-nitrocatechol by *Burkholderia cepacia*: A plasmid-encoded novel pathway. *J. Appl. Microbiol.*, 88:764–772.
25. Cleasby, A., Wonacott, A., Skarzynski, T., Hubbard, R.E., Davies, G.J., Proudfoot, A.E., Bernard, A.R., Payton, M.A., and Wells, T.N., 1996, The x-ray crystal structure of phosphomannose isomerase from *Candida albicans* at 1.7 angstrom resolution. *Nat. Struct. Biol.*, 3:470–479.
26. Cooper, R.A. and Skinner, M.A., 1980, Catabolism of 3- and 4-hydroxyphenylacetate by the 3,4-dihydroxyphenylacetate pathway in *Escherichia coli*. *J. Bacteriol.*, 143:302–306.
27. Crawford, R.L., 1976, Pathways of 4-hydroxybenzoate degradation among species of *Bacillus*. *J. Bacteriol.*, 127:204–210.
28. Crawford, R.L., Hutton, S.W., and Chapman, P.J., 1975, Purification and properties of gentisate 1,2-dioxygenase from *Moraxella osloensis*. *J. Bacteriol.*, 121:794–799.
29. Dagley, S., 1978, Determinants of biodegradability. *Q. Rev. Biophys.*, 11:577–602.
30. Dagley, S., 1986, Biochemistry of aromatic hydrocarbon degradation in Pseudomonads. In J.R. Sokatch and J.L. Ornston (eds), *The Bacteria*, vol. 10, pp. 527–555. Academic Press Inc., Orlando, FL.
31. Dai, S., Vaillancourt, F.H., Maaroufi, H., Drouin, N.M., Neau, D.B., Snieckus, V., Bolin, J.T., and Eltis, L.D., 2002, Identification and analysis of a bottleneck in PCB biodegradation. *Nat. Struct. Biol.*, 9:934–939.
32. Dai, Y., Wensink, P.C., and Abeles, R.H., 1999, One protein, two enzymes. *J. Biol. Chem.*, 274:1193–1195.
33. Daubaras, D.L., Hersherberger, C.D., Kitano, K., and Chakrabarty, A.M., 1995, Sequence analysis of a gene cluster involved in metabolism of 2,4,5-trichlorophenoxyacetic acid by *Burkholderia cepacia* AC1100. *Appl. Environ. Microbiol.*, 61:1279–1289.
34. Davis, J.K., He, Z., Somerville, C.C., and Spain, J.C., 1999, Genetic and biochemical comparison of 2-aminophenol 1,6-dioxygenase of *Pseudomonas pseudoalcaligenes* JS45 to meta-cleavage dioxygenases: Divergent evolution of 2-aminophenol meta-cleavage pathway. *Arch. Microbiol.*, 172:330–339.
35. Davis, M.I., Wasinger, E.C., Decker, A., Pau, M.Y.M., Vaillancourt, F.H., Bolin, J.T., Eltis, L.D., Hedman, B., Hodgson, K.O., and Solomon, E.I., 2003, Spectroscopic and electronic structure studies of 2,3-dihydroxybiphenyl 1,2-dioxygenase: O₂ reactivity of the non-heme ferrous site in extradiol dioxygenases. *J. Am. Chem. Soc.*, 125:11214–11227.
36. DeLano, W.L., 2002, *The PyMOL Molecular Graphics System*. DeLano Scientific, San Carlos, CA.
37. Dumas, P., Bergdoll, M., Cagnon, C., and Masson, J.M., 1994, Crystal structure and site-directed mutagenesis of a bleomycin resistance protein and their significance for drug sequestering. *EMBO J.*, 13:2483–2492.
38. Dunwell, J.M., Culham, A., Carter, C.E., Sosa-Aguirre, C.R., and Goodenough, P.W., 2001, Evolution of functional diversity in the cupin superfamily. *Trends Biochem. Sci.*, 26:740–746.
39. Dunwell, J.M., Khuri, S., and Gane, P.J., 2000, Microbial relatives of the seed storage proteins of higher plants: Conservation of structure and diversification of function during evolution of the cupin superfamily. *Microbiol. Mol. Biol. Rev.*, 64:153–179.
40. Elgren, T.E., Orville, A.M., Kelly, K.A., Lipscomb, J.D., Ohlendorf, D.H., and Que, L. Jr, 1997, Crystal structure and resonance Raman studies of protocatechuate 3,4-dioxygenase complexed with 3,4-dihydroxyphenylacetate. *Biochemistry*, 36:11504–11513.
41. Eltis, L.D. and Bolin, J.T., 1996, Evolutionary relationships among extradiol dioxygenases. *J. Bacteriol.*, 178:5930–5937.

42. Eltis, L.D., Hofmann, B., Hecht, H.-J., Lunsdorf, H., and Timmis, K.N., 1993, Purification and crystallization of 2,3-dihydroxybiphenyl 1,2-dioxygenase. *J. Biol. Chem.*, 268:2727–2732.
43. Fetzner, S., 2002, Oxygenases without requirement for cofactors or metal ions. *Appl. Microbiol. Biotechnol.*, 60:243–257.
44. Flatmark, T. and Stevens, R.C., 1999, Structural insight into the aromatic amino acid hydroxylases and their disease-related mutant forms. *Chem. Rev.*, 99:2137–2160.
45. Frazee, R.W., Orville, A.M., Dolbeare, K.B., Yu, H., Ohlendorf, D.H., and Lipscomb, J.D., 1998, The axial tyrosinate Fe^{3+} ligand in protocatechuate 3,4-dioxygenase influences substrate binding and product release: Evidence for new reaction cycle intermediates. *Biochemistry*, 37:2131–2144.
46. Fusetti, F., Schroter, K.H., Steiner, R.A., van Noort, P.I., Pijning, T., Rozeboom, H.J., Kalk, K.H., Egmond, M.R., and Dijkstra, B.W., 2002, Crystal structure of the copper-containing quercetin 2,3-dioxygenase from *Aspergillus japonicus*. *Structure*, 10:259–268.
47. Gaal, A. and Neujahr, H.Y., 1979, Metabolism of phenol and resorcinol in *Trichosporon cutaneum*. *J. Bacteriol.*, 137:13–21.
48. Gerlt, J.A. and Babbitt, P.C., 2001, Divergent evolution of enzymatic function: Mechanistically diverse superfamilies and functionally distinct suprafamilies. *Ann. Rev. Biochem.*, 70:209–246.
49. Gescher, J., Zaar, A., Mohamed, M., Schagger, H., and Fuchs, G., 2002, Genes coding for a new pathway of aerobic benzoate metabolism in *Azoarcus evansii*. *J. Bacteriol.*, 184:6301–6315.
50. Gibello, A., Ferrer, E., Martin, M., and Garrido-Pertierra, A., 1994, 3,4-Dihydroxyphenylacetate 2,3-dioxygenase from *Klebsiella pneumoniae*, a Mg^{2+} -containing dioxygenase involved in aromatic catabolism. *Biochem. J.*, 301:145–150.
51. Gibson, D.T., Koch, J.R., and Kallio, R.E., 1968, Oxidative degradation of aromatic hydrocarbons by microorganisms. I. Enzymatic formation of catechol from benzene. *Biochemistry*, 7:2653–2662.
52. Grund, E., Denecke, B., and Eichenlaub, R., 1992, Naphthalene degradation via salicylate and gentisate by *Rhodococcus* sp. strain B4. *Appl. Environ. Microbiol.*, 58:1874–1877.
53. Hamilton, A.J., Lycett, G.W., and Grierson, D., 1990, Antisense gene that inhibits synthesis of the hormone ethylene in transgenic plants. *Nature*, 346:284–287.
54. Han, S., Eltis, L.D., Timmis, K.N., Muchmore, S.W., and Bolin, J.T., 1995, Crystal structure of the biphenyl-cleaving extradiol dioxygenase from a PCB-degrading pseudomonad. *Science*, 270:976–980.
55. Happe, B., Eltis, L.D., Poth, H., Hedderich, R., and Timmis, K.N., 1993, Characterization of 2,2',3'-trihydroxybiphenyl dioxygenase, an extradiol dioxygenase from the dibenzofuran- and dibenzo-p-dioxin-degrading bacterium *Sphingomonas* sp. strain RW1. *J. Bacteriol.*, 175:7313–7320.
56. Harayama, S. and Rekik, M., 1989, Bacterial aromatic ring-cleavage enzymes are classified into two different gene families. *J. Biol. Chem.*, 264:15328–15333.
57. Harayama, S., Kok, M., and Neidle, E.L., 1992, Functional and evolutionary relationships among diverse oxygenases. *Annu. Rev. Microbiol.*, 46:565–601.
58. Harpel, M.R. and Lipscomb, J.D., 1990, Gentisate 1,2-dioxygenase from pseudomonas. Purification, characterization, and comparison of the enzymes from *Pseudomonas testosteroni* and *Pseudomonas acidovorans*. *J. Biol. Chem.*, 265:6301–6311.
59. Hatta, T., Mukerjee-Dhar, G., Damborsky, J., Kiyohara, H., and Kimbara, K., 2003, Characterization of a novel thermostable Mn(II)-dependent 2,3-dihydroxybiphenyl 1,2-dioxygenase from a PCB and naphthalene-degrading *Bacillus* sp. JF8. *J. Biol. Chem.*, 278:21483–21492.

60. Hayaishi, O. and Hashimoto, K., 1950, Pyrocatecase, a new enzyme catalyzing oxidative breakdown of pyrocatechin. *J. Biochem.*, 37:371–374.
61. Hegg, E.L. and Que, L. Jr, 1997, The 2-His-1-carboxylate facial triad—an emerging structural motif in mononuclear non-heme iron(II) enzymes. *Eur. J. Biochem.*, 250:625–629.
62. Heiss, G., Stolz, A., Kuhm, A.E., Müller, C., Klein, J., Altenbuchner, J., and Knackmuss, H.-J., 1995, Characterization of a 2,3-dihydroxybiphenyl dioxygenase from the naphthalene-sulfonate-degrading bacterium strain BN6. *J. Bacteriol.*, 177:5865–5871.
63. Hewitson, K.S., McNeill, L.A., Riordan, M.V., Tian, Y.-M., Bullock, A.N., Welford, R.W., Elkins, J.M., Oldham, N.J., Bhattacharya, S., Gleadle, J.M., Ratcliffe, P.J., Pugh, C.W., and Schofield, C.J., 2002, Hypoxia-inducible factor (HIF) asparagine hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. *J. Biol. Chem.*, 277:26351–26355.
64. Hintner, J.P., Lechner, C., Riegert, U., Kuhm, A.E., Storm, T., Reemtsma, T., and Stolz, A., 2001, Direct ring fission of salicylate by a salicylate 1,2-dioxygenase activity from *Pseudaminobacter salicylatoxidans*. *J. Bacteriol.*, 183:6936–6942.
65. Hopper, D.J. and Taylor, D.G., 1975, Pathways for the degradation of *m*-cresol and *p*-cresol by *Pseudomonas putida*. *J. Bacteriol.*, 122:1–6.
66. Hori, K., Hashimoto, T., and Nozaki, M., 1973, Kinetic studies on the reaction mechanism of dioxygenases. *J. Biochem.*, 74:375–384.
67. Hughes, E.J. and Bayly, R.C., 1983, Control of catechol meta-cleavage pathway in *Alcaligenes eutrophus*. *J. Bacteriol.*, 154:1363–1370.
68. Hugo, N., Meyer, C., Armengaud, J., Gaillard, J., Timmis, K.N., and Jouanneau, Y., 2000, Characterization of three XylT-like [2Fe-2S] ferredoxins associated with catabolism of cresols or naphthalene: Evidence for their involvement in catechol dioxygenase reactivation. *J. Bacteriol.*, 182:5580–5585.
69. Imbeault, N.Y.R., Powlowski, J.B., Colbert, C.L., Bolin, J.T., and Eltis, L.D., 2000, Steady-state kinetic characterization and crystallization of a polychlorinated biphenyl-transforming dioxygenase. *J. Biol. Chem.*, 275:12430–12437.
70. Iwabuchi, T. and Harayama, S., 1998, Biochemical and molecular characterization of 1-hydroxy-2-naphthoate dioxygenase from *Nocardioideis* sp. KP7. *J. Biol. Chem.*, 273:8332–8336.
71. Jain, R.K., Dreisbach, J.H., and Spain, J.C., 1994, Biodegradation of *p*-nitrophenol via 1,2,4-benzenetriol by an *Arthrobacter* sp. *Appl. Environ. Microbiol.* 60:3030–3032.
72. Jeffrey, A.M., Yeh, H.J., Jerina, D.M., Patel, T.R., Davey, J.F., and Gibson, D.T., 1975, Initial reactions in the oxidation of naphthalene by *Pseudomonas putida*. *Biochemistry*, 14:575–584.
73. Kabisch, M. and Fortnagel, P., 1990, Nucleotide sequence of metapyrocatechase I (catechol 2,3-oxygenase I) gene *mpcI* from *Alcaligenes eutrophus* JMP222. *Nucleic Acids Res.*, 18:3405–3406.
74. Kauppi, B., Lee, K., Carredano, E., Parales, R.E., Gibson, D.T., Eklund, H., and Ramaswamy, S., 1998, Structure of an aromatic-ring-hydroxylating dioxygenase-naphthalene 1,2-dioxygenase. *Structure*, 6:571–586.
75. Keyser, P., Pujar, B.G., Eaton, R.W., and Ribbons, D.W., 1976, Biodegradation of the phthalates and their esters by bacteria. *Environ. Health Perspect.*, 18:159–166.
76. Kita, A., Kita, S., Fujisawa, I., Inaka, K., Ishida, T., Horiike, K., Nozaki, M., and Miki, K., 1999, An archetypical extradiol-cleaving catecholic dioxygenase: The crystal structure of catechol 2,3-dioxygenase (metapyrocatechase) from *Pseudomonas putida* mt-2. *Structure*, 7:25–34.
77. Klages, U., Markus, A., and Lingens, F., 1981, Degradation of 4-chlorophenylacetic acid by a *Pseudomonas* species. *J. Bacteriol.*, 146:64–68.
78. Klecka, G.M. and Gibson, D.T., 1981, Inhibition of catechol 2,3-dioxygenase from *Pseudomonas putida* by 3-chlorocatechol. *Appl. Environ. Microbiol.*, 41:1159–1165.

79. Kobayashi, T., Ishida, T., Horiike, K., Takahara, Y., Numao, N., Nakazawa, A., Nakazawa, T., and Nozaki, M., 1995, Overexpression of *Pseudomonas putida* catechol 2,3-dioxygenase with high specific activity by genetically engineered *Escherichia coli*. *J. Biochem.*, 117:614–622.
80. Kojima, Y., Itada, N., and Hayaishi, O., 1961, Metapyrocatechase: A new catechol-cleaving enzyme. *J. Biol. Chem.*, 236:2223–2228.
81. Kraulis, P.J., 1991, MOLSCRIPT: A program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr.*, 24:945–949.
82. Kukor, J.J. and Olsen, R.H., 1996, Catechol 2,3-dioxygenases functional in oxygen-limited (hypoxic) environments. *Appl. Environ. Microbiol.*, 62:1728–1740.
83. La Du, B.N., Zannoni, V.G., Laster, L., and Seegmiller, J.E., 1958, Nature of the defect in tyrosine metabolism in alcaptonuria. *J. Biol. Chem.*, 230:251–260.
84. Lah, M.S., Dixon, M.M., Patridge, K.A., Stallings, W.C., Fee, J.A., and Ludwig, M.L., 1995, Structure-function in *Escherichia coli* iron superoxide dismutase: Comparisons with the manganese enzyme from *Thermus thermophilus*. *Biochemistry*, 34:1646–1660.
85. Lendenmann, U. and Spain, J.C., 1996, 2-aminophenol 1,6-dioxygenase: A novel aromatic ring cleavage enzyme purified from *Pseudomonas pseudoalcaligenes* JS45. *J. Bacteriol.*, 178:6227–6232.
86. Lin, G., Reid, G., and Bugg, T.D.H., 2001, Extradial oxidative cleavage of catechols by ferrous and ferric complexes of 1,4,7-triazacyclononane: Insight into the mechanism of the extradiol catechol dioxygenases. *J. Am. Chem. Soc.*, 123:5030–5039.
87. Mabrouk, P.A., Orville, A.M., Lipscomb, J.D., and Solomon, E.I., 1991, Variable-temperature variable-field magnetic circular dichroism studies of the iron(II) active site in metapyrocatechase: Implications for the molecular mechanism of extradiol dioxygenases. *J. Am. Chem. Soc.*, 113:4053–4061.
88. Mars, A.E., Kasberg, T., Kaschabek, S.R., van Agteren, M.H., Janssen D.B., and Reineke, W., 1997, Microbial degradation of chloroaromatics: Use of the meta-cleavage pathway for mineralization of chlorobenzene. *J. Bacteriol.*, 179:4530–4537.
89. Mars, A.E., Kingma, J., Kaschabek, S.R., Reineke, W., and Janssen, D.B., 1999, Conversion of 3-chlorocatechol by various catechol 2,3-dioxygenases and sequence analysis of the chlorocatechol dioxygenase region of *Pseudomonas putida* GJ31. *J. Bacteriol.*, 181:1309–1318.
90. Mashetty, S.B., Manohar, S., and Karegoudar, T.B., 1996, Degradation of 3-hydroxybenzoic acid by a *Bacillus* species. *Indian J. Biochem. Biophys.*, 33:145–148.
91. Mattevi, A., Fraaije, M.W., Mozzarelli, A., Olivi, L., Coda, A., and van Berkel, W.J., 1997, Crystal structures and inhibitor binding in the octameric flavoenzyme vanillyl-alcohol oxidase: The shape of the active-site cavity controls substrate specificity. *Structure*, 5:907–920.
92. McCarthy, A.A., Baker, H.M., Shewry, S.C., Patchett, M.L., and Baker, E.N., 2001, Crystal structure of methylmalonyl-coenzyme A epimerase from *P. shermanii*: A novel enzymatic function on an ancient metal binding scaffold. *Structure*, 9:637–646.
93. McMurry, J., 1992, *Organic Chemistry*, 3rd edn, Brooks/Cole, Pacific Grove, CA.
94. Merritt, E.A. and Bacon, D.J., 1997, Raster3D: Photorealistic molecular graphics. *Methods Enzymol.*, 277:505–524.
95. Minor, W., Steczko, J., Stec, B., Otwinowski, Z., Bolin, J.T., Walter, R., and Axelrod, B., 1996, Crystal structure of soybean lipoxygenase L-1 at 1.4 Å resolution. *Biochemistry*, 35:10687–10701.
96. Mitchell, R.A., Kang, H.H., and Henderson, L.M., 1963, Inactivation during functioning of 3-hydroxyanthranilate oxidase resulting from oxidation of bound ferrous iron. *J. Biol. Chem.*, 238:1151–1155.
97. Miyauchi, K., Adachi, Y., Nagata, Y., and Takagi, M., 1999, Cloning and sequencing of a novel meta-cleavage dioxygenase gene whose product is involved in degradation of gamma-hexachlorocyclohexane in *Sphingomonas paucimobilis*. *J. Bacteriol.*, 181:6712–6719.

98. Mohamed, M., E., Zaar, A., Ebenau-Jehle, C., and Fuchs, G. 2001, Reinvestigation of a new type of aerobic benzoate metabolism in the proteobacterium *Azoarcus evansii*. *J. Bacteriol.*, 183:1899–1908.
99. Muraki, T., Taki, M., Hasegawa, Y., Iwaki, H., and Lau, P.C., 2003, Prokaryotic homologs of the eukaryotic 3-hydroxyanthranilate 3,4-dioxygenase and 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase in the 2-nitrobenzoate degradation pathway of *Pseudomonas fluorescens* strain KU-7. *Appl. Environ. Microbiol.*, 69:1564–1572.
100. Murray, K., Duggleby, C.J., Sala-Trepat, J.M., and Williams, P.A., 1972, The metabolism of benzoate and methylbenzoates via the meta-cleavage pathway by *Pseudomonas arvilla* mt-2. *Eur. J. Biochem.*, 28:301–310.
101. Nozaki, M., Kagamiyama, H., and Hayaishi, O., 1963, Metapyrocatechase. I. Purification, crystallization, and some properties. *Biochem. Z.*, 338:582–590.
102. Nozaki, M., Katsushiko, K., Nakazawa, T., Kotani, S., and Hayaishi, O., 1968, Metapyrocatechase II. The role of iron and sulfhydryl groups. *J. Biol. Chem.*, 243: 2682–2690.
103. Ohlendorf, D.H. and Vetting, M.W., 2001, Protocatechuate 3,4-dioxygenase. In A. Messerschmidt, R. Huber, T. Poulos, and K. Wieghardt (eds), *Handbook of Metalloproteins*, pp. 622–631. John Wiley & Sons, Chichester, UK.
104. Ohlendorf, D.H., Lipscomb, J.D., and Weber, P.C., 1988, Structure and assembly of protocatechuate 3,4-dioxygenase. *Nature*, 336:403–405.
105. Ohlendorf, D.H., Orville, A.M., and Lipscomb, J.D., 1994, Structure of protocatechuate 3,4-dioxygenase from *Pseudomonas aeruginosa* at 2.15 Å resolution. *J. Mol. Biol.*, 244:586–608.
106. Ono, K., Nozaki, M., and Hayaishi, O., 1970, Purification and some properties of protocatechuate 4,5-dioxygenase. *Biochim. Biophys. Acta*, 220:224–238.
107. Orville, A.M. and Lipscomb, J.D., 1997, Cyanide and nitric oxide binding to reduced protocatechuate 3,4-dioxygenase: Insight into the basis for order-dependent ligand binding by intradiol catecholic dioxygenases. *Biochemistry*, 36:14044–14055.
108. Orville, A.M., Lipscomb, J.D., and Ohlendorf, D.H., 1997, Crystal structures of substrate and substrate analog complexes of protocatechuate 3,4-dioxygenase: Endogenous Fe³⁺ ligand displacement in response to substrate binding. *Biochemistry*, 36:10052–10066.
109. Parli, C.J., Krieter, P., and Schmidt, B., 1980, Metabolism of 6-chlorotryptophan to 4-chloro-3-hydroxyanthranilic acid: A potent inhibitor of 3-hydroxyanthranilic acid oxidase. *Arch. Biochem. Biophys.*, 203:161–166.
110. Pascal, R.A. and Huang, D.-S., 1987, Mechanism-based inactivation of catechol 2,3-dioxygenase by 3-[(methylthio)methyl]catechol. *J. Am. Chem. Soc.*, 109:2854–2855.
111. Pochapsky, T.C., Pochapsky, S.S., Ju, T., Mo, H., Al-Mjeni, F., and Maroney, M.J., 2002, Modeling and experiment yields the structure of acireductone dioxygenase from *Klebsiella pneumoniae*. *Nat. Struct. Biol.* 9:966–972.
112. Polissi, A. and Harayama, S., 1993, *In vivo* reactivation of catechol 2,3-dioxygenase mediated by a chloroplast-type ferredoxin: A bacterial strategy to expand the substrate specificity of aromatic degradative pathways. *EMBO J.*, 12:3339–3347.
113. Priefert, H., Rabenhorst, J., and Steinbuchel, A., 1997, Molecular characterization of genes of *Pseudomonas* sp. strain HR199 involved in bioconversion of vanillin to protocatechuate. *J. Bacteriol.*, 179:2595–2607.
114. Que, L. Jr and Epstein, R.M., 1981, Resonance Raman studies on protocatechuate 3,4-dioxygenase-inhibitor complexes. *Biochemistry*, 20:2545–2549.
115. Que, L. Jr and Ho, R.Y.N., 1996, Dioxygen activation by enzymes with mononuclear non-heme iron active sites. *Chem. Rev.*, 96:2607–2624.
116. Que, L. Jr and Reynolds, M.F., 2000, Manganese(II)-dependent extradiol-cleaving catechol dioxygenases. *Met. Ions Biol. Syst.*, 37:505–525.

117. Que, L. Jr., 2000, One motif—many different reactions. *Nat. Struct. Biol.*, 7:182–184.
118. Que, L. Jr., Lipscomb, J.D., Münck, E., and Wood, J.M., 1977, Protocatechuate 3,4-dioxygenase. Inhibitor studies and mechanistic implications. *Biochim. Biophys. Acta*, 485:60–74.
119. Riegert, U., Heiss, G., Fischer, P., and Stolz, A., 1998, Distal cleavage of 3-chlorocatechol by an extradiol dioxygenase to 3-chloro-2-hydroxymuconic semialdehyde. *J. Bacteriol.*, 180:2849–2853.
120. Roach, P.L., Clifton, I.J., Hensgens, C.M., Shibata, N., Schofield, C.J., Hajdu, J., and Baldwin, J.E., 1997, Structure of isopenicillin N synthase complexed with substrate and the mechanism of penicillin formation. *Nature*, 387:827–830.
121. Rojo, F., Pieper, D.H., Engesser, K.H., Knackmuss, H.-J., and Timmis, K.N., 1987, Assemblage of *ortho* cleavage route for simultaneous degradation of chloro- and methylaromatics. *Science*, 238:1395–1398.
122. Roper, D.I. and Cooper, R.A., 1990, Subcloning and nucleotide sequence of the 3,4-dihydroxyphenylacetate (homoprotocatechuate) 2,3-dioxygenase gene from *Escherichia coli* C. *FEBS Lett.*, 275:53–57.
123. Sanvoisin, J., Langley, G.J., and Bugg, T.D.H., 1995, Mechanism of extradiol catechol dioxygenases: Evidence for a lactone intermediate in the 2,3-dihydroxyphenylpropionate 1,2-dioxygenase reaction. *J. Am. Chem. Soc.*, 117:7836–7837.
124. Sato, N., Uragami, Y., Nishizaki, T., Takahashi, Y., Sazaki, G., Sugimoto, K., Nonaka, T., Masai, E., Fukuda, M., and Senda, T., 2002, Crystal structures of the reaction intermediate and its homologue of an extradiol-cleaving catecholic dioxygenase. *J. Mol. Biol.*, 321:621–636.
125. Schwarcz, R., Okuno, E., White, R.J., Bird, E.D., and Whetsell, W.O. Jr, 1988, 3-Hydroxyanthranilate oxygenase activity is increased in the brains of Huntington disease victims. *Proc. Natl. Acad. Sci. USA*, 85:4079–4081.
126. Senda, T., Sugiyama, K., Narita, H., Yamamoto, T., Kimbara, K., Fukuda, M., Sato, M., Yano, K., and Mitsui, Y., 1996, Three-dimensional structures of free form and two substrate complexes of an extradiol ring-cleavage type dioxygenase, the BphC enzyme from *Pseudomonas* sp. strain KKS102. *J. Mol. Biol.*, 255:735–752.
127. Serre, L., Sailland, A., Sy, D., Boudec, P., Rolland, A., Pebay-Peyroula, E., and Cohen-Addad, C., 1999, Crystal structure of *Pseudomonas fluorescens* 4-hydroxyphenylpyruvate dioxygenase: An enzyme involved in the tyrosine degradation pathway. *Structure*, 7:977–988.
128. Shu, L., Chiou, Y.-M., Orville, A.M., Miller, M.A., Lipscomb, J.D., and Que, L. Jr, 1995, X-ray absorption spectroscopic studies of the Fe(II) active site of catechol 2,3-dioxygenase. Implications for the extradiol cleavage mechanism. *Biochemistry*, 34:6649–6659.
129. Solomon, E.I., Brunold, T.C., Davis, M.I., Kemsley, J.N., Lee, S.-K., Lehnert, N., Neese, F., Skulan, A.J., Yang, Y.-S., and Zhou, J., 2000, Geometric and electronic structure/function correlations in non-heme iron enzymes. *Chem. Rev.*, 100:235–349.
130. Spence, E.L., Kawamukai, M., Sanvoisin, J., Braven, H., and Bugg, T.D.H., 1996, Catechol dioxygenases from *Escherichia coli* (MhpB) and *Alcaligenes eutrophus* (MpcI): Sequence analysis and biochemical properties of a third family of extradiol dioxygenases. *J. Bacteriol.*, 178:5249–5256.
131. Spence, E.L., Langley, G.J., and Bugg, T.D.H., 1996, Cis-Trans isomerization of a cyclopropyl radical trap catalyzed by extradiol catechol dioxygenases: Evidence for a semiquinone intermediate. *J. Am. Chem. Soc.*, 118:8336–8343.
132. Stanier, R.Y. and Ingraham, J.L., 1954, Protocatechuic acid oxidase. *J. Biol. Chem.*, 210:799–808.
133. Stolz, A., Nortemann, B., and Knackmuss, H.-J., 1992, Bacterial metabolism of 5-aminosalicylic acid. Initial ring cleavage. *Biochem. J.*, 282:675–680.

134. Suda, S. and Takeda, Y., 1950, Metabolism of tyrosine 1. Application of successive adaptation of bacteria for the analysis of the enzymatic breakdown of tyrosine. *J. Biochem.*, 37:375–378.
135. Sugimoto, K., Senda, T., Aoshima, H., Masai, E., Fukuda, M., and Mitsui, Y., 1999, Crystal structure of an aromatic ring opening dioxygenase LigAB, a protocatechuate 4,5-dioxygenase, under aerobic conditions. *Structure*, 7:953–965.
136. Takenaka, S., Murakami, S., Shinke, R., Hatakeyama, K., Yukawa, H., and Aoki, K., 1997, Novel genes encoding 2-aminophenol 1,6-dioxygenase from *Pseudomonas* species AP-3 growing on 2-aminophenol and catalytic properties of the purified enzyme. *J. Biol. Chem.*, 272:14727–14732.
137. Timmis, K.N., Steffan, R.J., and Unterman, R., 1994, Designing microorganisms for the treatment of toxic wastes. *Annu. Rev. Microbiol.*, 48:525–557.
138. Titus, G.P., Mueller, H.A., Burgner, J., Rodriguez de Cordoba, S., Penalva, M.A., and Timm, D.E., 2000, Crystal structure of human homogentisate dioxygenase. *Nat. Struct. Biol.*, 7:542–546.
139. Tropel, D., Meyer, C., Armengaud, J., and Jouanneau, Y., 2002, Ferredoxin-mediated reactivation of the chlorocatechol 2,3-dioxygenase from *Pseudomonas putida* GJ31. *Arch. Microbiol.*, 177:345–351.
140. True, A.E., Orville, A.M., Pearce, L.L., Lipscomb, J.D., and Que, L. Jr, 1990, An EXAFS study of the interaction of substrate with the ferric active site of protocatechuate 3,4-dioxygenase. *Biochemistry*, 29:10847–10854.
141. Tyson, C.A., 1975, 4-Nitrocatechol as a colorimetric probe for non-heme iron dioxygenases. *J. Biol. Chem.*, 250:1765–1770.
142. Uragami, Y., Senda, T., Sugimoto, K., Sato, N., Nagarajan, V., Masai, E., Fukuda, M., and Mitsui, Y., 2001, Crystal structures of substrate free and complex forms of reactivated BphC, an extradiol type ring-cleavage dioxygenase. *J. Inorg. Biochem.*, 83:269–279.
143. Vaillancourt, F.H., Barbosa, C.J., Spiro, T.G., Bolin, J.T., Blades, M.W., Turner, R.F.B., and Eltis, L.D., 2002, Definitive evidence for monoanionic binding of 2,3-dihydroxybiphenyl to 2,3-dihydroxybiphenyl 1,2-dioxygenase from UV resonance Raman spectroscopy, UV/Vis absorption spectroscopy, and crystallography. *J. Am. Chem. Soc.*, 124:2485–2496.
144. Vaillancourt, F.H., Han, S., Fortin, P.D., Bolin, J.T., and Eltis, L.D., 1998, Molecular basis for the stabilization and inhibition of 2, 3-dihydroxybiphenyl 1,2-dioxygenase by *t*-butanol. *J. Biol. Chem.*, 273:34887–34895.
145. Vaillancourt, F.H., Haro, M.A., Drouin, N.M., Karim, Z., Maaroufi, H., and Eltis, L.D., 2003, Characterization of extradiol dioxygenases from a polychlorinated biphenyl-degrading strain that possess higher specificities for chlorinated metabolites. *J. Bacteriol.*, 185:1253–1260.
146. Vaillancourt, F.H., Labbé, G., Drouin, N.M., Fortin, P.D., and Eltis, L.D., 2002, The mechanism-based inactivation of 2,3-dihydroxybiphenyl 1,2-dioxygenase by catecholic substrates. *J. Biol. Chem.*, 277:2019–2027.
147. Vølgard, K., van Scheltinga, A.C., Lloyd, M.D., Hara, T., Ramaswamy, S., Perrakis, A., Thompson, A., Lee, H.J., Baldwin, J.E., Schofield, C.J., Hajdu, J., and Andersson, I., 1998, Structure of a cephalosporin synthase. *Nature*, 394:805–809.
148. Vescia, A. and Di Prisco, G., 1962, Studies on purified 3-hydroxyanthranilic acid oxidase. *J. Biol. Chem.*, 237:2318–2324.
149. Vetting, M.W. and Ohlendorf, D.H., 2000, The 1.8 Å crystal structure of catechol 1,2-dioxygenase reveals a novel hydrophobic helical zipper as a subunit linker. *Structure*, 8:429–440.
150. Vetting, M.W., D'Argenio, D.A., Ornston, L.N., and Ohlendorf, D.H., 2000, Structure of *Acinetobacter* strain ADP1 protocatechuate 3, 4-dioxygenase at 2.2 Å resolution: Implications for the mechanism of an intradiol dioxygenase. *Biochemistry*, 39:7943–7955.

151. Walsh, J.L., Wu, H.-Q., Ungerstedt, U., and Schwarcz, R., 1994, 4-Chloro-3-hydroxyanthranilate inhibits quinolinate production in the rat hippocampus *in vivo*. *Brain Res. Bull.*, 33:513–516.
152. Walsh, T.A., Ballou, D.P., Mayer, R., and Que, L. Jr, 1983, Rapid reaction studies on the oxygenation reactions of catechol dioxygenase. *J. Biol. Chem.*, 258:14422–14427.
153. Wang, Y.Z. and Lipscomb, J.D. 1997, Cloning, overexpression, and mutagenesis of the gene for homoprotocatechuate 2,3-dioxygenase from *Brevibacterium fuscum*. *Protein Expr. Purif.*, 10:1–9.
154. Wasinger, E.C., Davis, M.I., Pau, M.Y.M., Orville, A.M., Zaleski, J.M., Hedman, B., Lipscomb, J.D., Hodgson, K.O., and Solomon, E.I., 2003, Spectroscopic studies of the effect of ligand donor strength on the Fe-NO bond in triradiol dioxygenases. *Inorg. Chem.*, 42:365–376.
155. Wasserfallen, A., 1989, Biochemical and genetical study of the specificity of catechol 2,3-dioxygenase from *Pseudomonas putida*. Ph.D. thesis. University of Geneva.
156. Whiting, A.K., Boldt, Y.R., Hendrich, M.P., Wackett, L.P., and Que, L. Jr, 1996, Manganese(II)-dependent extradiol-cleaving catechol dioxygenase from *Arthrobacter globiformis* CM-2. *Biochemistry*, 35:160–170.
157. Winfield, C.J., Al-Mahrizy, Z., Gravestock, M., and Bugg, T.D.H., 2000, Elucidation of the catalytic mechanisms of the non-haem iron-dependent catechol dioxygenases: Synthesis of carba-analogues for hydroperoxide reaction intermediates. *J. Chem. Soc., Perkin Trans. 1*, 2000:3277–3289.
158. Wolgel, S.A. and Lipscomb, J.D., 1990, Protocatechuate 2,3-dioxygenase from *Bacillus macerans*. *Methods Enzymol.*, 188:95–101.
159. Woo, E.J., Dunwell, J.M., Goodenough, P.W., Marvier, A.C., and Pickersgill, R.W., 2000, Germin is a manganese containing homohexamer with oxalate oxidase and superoxide dismutase activities. *Nat. Struct. Biol.*, 7:1036–1040.
160. Xu, L., Resing, K., Lawson, S.L., Babbitt, P.C., and Copley, S.D., 1999, Evidence that pcpA encodes 2,6-dichlorohydroquinone dioxygenase, the ring cleavage enzyme required for pentachlorophenol degradation in *Sphingomonas chlorophenolica* strain ATCC 39723. *Biochemistry*, 38:7659–7669.
161. Yamaguchi, K., Hosokawa, Y., Kohashi, N., Kori, Y., Sakakibara, S., and Ueda, I., 1978, Rat liver cysteine dioxygenase (cysteine oxidase). Further purification, characterization, and analysis of the activation and inactivation. *J. Biochem.*, 83:479–491.
162. Zaar, A., Eisenreich, W., Bacher, A., and Fuchs, G., 2001, A novel pathway of aerobic benzoate catabolism in the bacteria *Azoarcus evansii* and *Bacillus stearothermophilus*. *J. Biol. Chem.*, 276:24997–25004.
163. Zaborina, O., Latus, M., Eberspacher, J., Golovleva, L.A., and Lingens, F., 1995, Purification and characterization of 6-chlorohydroxyquinol 1,2-dioxygenase from *Streptomyces rochei* 303: Comparison with an analogous enzyme from *Azotobacter* sp. strain GP1. *J. Bacteriol.*, 177:229–234.
164. Zhang, Z., Ren, J., Stammers, D.K., Baldwin, J.E., Harlos, K., and Schofield, C.J., 2000, Structural origins of the selectivity of the trifunctional oxygenase clavaminic acid synthase. *Nat. Struct. Biol.*, 7:127–133.
165. Zhao, G., Xia, T., Song, J., and Jensen, R.A., 1994, *Pseudomonas aeruginosa* possesses homologues of mammalian phenylalanine hydroxylase and 4 alpha-carbinolamine dehydratase/DCoH as part of a three-component gene cluster. *Proc. Natl. Acad. Sci. USA*, 91:1366–1370.

ALKANE DEGRADATION BY PSEUDOMONADS

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1. INTRODUCTION

Alkanes are highly reduced hydrocarbon compounds that constitute about 20–50% of crude oil. They are produced by geochemical processes from decaying plant and algal material, end up in the environment by natural oil seeps and human activities (oil-spills and run-off due to dispersed sources), and then disappear due to physical and biological degradation, a process estimated to amount to several million tons of alkanes per year^{4, 73}. A probably much larger quantity (predominantly long-chain linear compounds) is produced throughout the biosphere by living organisms (plants, animals, and bacteria) as a structural element, vapor barrier, waste product, defense mechanism, or chemoattractant. Consequently, these compounds are a reliable C- and energy source for microorganisms of many different genera belonging to the high- and low-(G+C) Gram-positives, and the α -, β -, and γ -Proteobacteria. Frequently mentioned genera are *Mycobacterium*, *Rhodococcus*, *Bacillus*, *Acinetobacter*, and *Pseudomonas*. Especially the last genus has played a prominent role in oil and alkane biodegradation studies as all lineages of the pseudomonads contain alkane-degrading species, they are easy to isolate and cultivate in the lab, not fastidious, (facultative) aerobes, grow well and as single cells in liquid

cultures. Although many organisms originally named 'Pseudomonas' have since been reclassified as α -, β -, and non-pseudomonad γ -Proteobacteria based on DNA-DNA hybridization⁸⁰ and 16S rRNA sequencing^{3, 54} the true pseudomonads of the lineages *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Pseudomonas putida*³ contain several well-characterized alkane-degrading isolates. The other major lineages of the pseudomonads; *Pseudomonas stutzeri*, *Pseudomonas chlororaphis*, and *Pseudomonas syringae*, also contain alkane-degraders, but these have not been characterized in detail.

This chapter focuses on the molecular genetics and enzymology of enzymes and other proteins that are directly or indirectly involved in terminal alkane oxidation to 1-alkanols by pseudomonads. Alternative pathways for the degradation of alkanes have been described, but these pathways are much less well documented. Similarly, the degradation pathways of branched and cyclic alkanes by pseudomonads have received much less attention, and will be discussed only briefly. 1-Alkanols formed by terminal oxygenation undergo further metabolic steps that are, strictly speaking, no longer unique to alkane degradation and are carried out by enzymes that are also present in pseudomonads that do not grow on alkanes. Therefore, we limit the discussion of downstream metabolism to enzymes that are co-induced with alkane hydroxylases.

2. ALKANE DEGRADING *PSEUDOMONAS* ISOLATES

Alkane-degrading microorganisms identified as *Bacterium fluorescens liquefaciens* (now *P. fluorescens*), *Bacterium pyocyaneum* (now *P. aeruginosa*), *Bacterium stutzeri* (now *P. stutzeri*) were isolated and shown to be numerous in garden soil and ditch water as early as 1913¹⁰⁴. In fact, alkane degraders are ubiquitous, and many pseudomonads able to grow on these compounds were described already prior to 1950^{37, 139}.

In the late 1950s, early 1960s of the last century, five alkane-degrading *Pseudomonas* strains, *P. aeruginosa* Sol 20, *P. aeruginosa* KSLA 473, *P. aeruginosa* 196 Aa, *P. aeruginosa* LA-P-6, and '*P. oleovorans*' ATCC 29347, were isolated on medium-chain length alkanes (C6–C10) in different regions of the world (for references and details, see Table 1). Early experiments with these isolates showed that alkanes are metabolized to CO₂ and water, and that 1-alkanols, aldehydes, and fatty acids are intermediates in the degradation pathway¹²⁷. In the following decades, the strains were studied with respect to their alkane degradation pathways, the substrate range and biochemical properties of the alkane-oxidizing enzymes, and the molecular genetics of the

Table 1. Alkane-degrading *Pseudomonas* isolates.

Strain name	Original name strain collection nr.	Source	Growth substrates	Properties of alkane oxidation enzymes	References
<i>P. putida</i> GPo1	<i>P. oleovorans</i> TF4-1L, ATCC 29347 <i>P. putida</i> strain 266 ATCC 17633	Chicago, USA, soil	C5-C13	Membrane-bound AH, rubredoxin; reductase	[8] [108, 121]
<i>P. putida</i> P1	—	Groningen, The Netherlands, gasoline-polluted soil	C6-C12	Membrane-bound AH, rubredoxin; reductase	[121]
<i>P. aeruginosa</i> KSLA 473	KSLA 473 (Shell Sittingbourne)	Amsterdam, The Netherlands, Y-harbor sediment	C6-C18	Three membrane-bound alkane hydroxylases	[115, 124, 130]
<i>P. aeruginosa</i> 196 Aa	NCIMB 9571	Louisiana, USA, fuel	C7-C16	Three membrane-bound alkane hydroxylases	[116, 124, 132]
<i>P. aeruginosa</i> Sol 20	NCIMB 8704	Marseille, France, oil-polluted soil	C6-C16	Three membrane-bound alkane hydroxylases	[6, 59, 124]
<i>P. aeruginosa</i> LA-P-6	ATCC 17423	Los Angeles, USA, hydrocarbon enrichment	C6-C16	Three membrane-bound alkane hydroxylases	[67, 108, 124]
<i>P. aeruginosa</i> <i>P. fluorescens</i> CHAO	NCIMB 9904 —	UK, soil Morens, Switzerland, soil	C12 C10-C32	Unknown, subterminal oxidation Membrane-bound alkane hydroxylase	[35] [100, 113]
<i>P. indica</i> IMT37	DSM 14015T	Gujarat, India, oil- polluted soil	C4-C10	Unrelated oxygenase	[78, 81]
<i>Pseudomonas</i> sp. 7/156	—	Antarctica, soil	?	Membrane-bound alkane hydroxylase	Genbank entry AY034587
<i>P. aureofaciens</i> RWTH 529	—	Aachen, Germany, soil	C10	Close homolog of <i>P. putida</i> GPo1 alkane hydroxylase	[133]
<i>P. denitrificans</i>	—	Japan	C6-C10	Three components (including rubredoxin and flavoprotein)	[60]
<i>P. oleovorans</i>	DSM1045T, ATCC 8062	Houston, cutting oil	(C8)	Unknown	[64, 89]

alkane-degradation pathways. Interestingly, in 1998 all five isolates turned out to possess enzyme systems that are virtually identical (more than 99% sequence identity)¹²⁴.

One of the five strains, the hexane-degrading soil isolate commonly known as *P. oleovorans* TF4-1L, GPo1, or ATCC 29347, has come to dominate the research on the applications, biochemistry, and molecular genetics of alkane oxidation by pseudomonads. It was first described in 1963⁸, and was at the time tentatively identified as a strain of *P. oleovorans*⁶⁴. After strain improvement for the production of epoxides⁹³, it was submitted to ATCC as *P. oleovorans* TF4-1L (ATCC 29347). However, in the authoritative paper of Stanier on the phylogeny of the pseudomonads¹⁰⁸, the original isolate, listed as strain 266, was classified as a *P. putida* biotype A²⁰. 'Strain 266' was also submitted to ATCC and is available but unlisted (ATCC 17633). As expected, the full-length 16S sequences of ATCC 29347 and 17633 were identical to each other¹²¹, and closely related to those of *P. putida* F1 and the *P. putida* biotype A type strain DSM291T (99.7% and 99.2% sequence identity, respectively). ATCC 29347 was clearly much more distantly related to other pseudomonads, including the type strain of *P. oleovorans*, DSM1045T (ATCC 8062), which was first described in 1941⁶⁴. Therefore, ATCC 29347, or GPo1, as the isolate is named in more recent publications, is now handled under the designation given by Stanier; *P. putida* GPo1¹²¹. In the original paper by Lee and Chandler, *P. oleovorans* (DSM1045T) was only shown to grow on naphthenic acids⁶⁴. Later it was reported to grow on *n*-octane⁸⁹, but this could not be confirmed¹⁰¹.

Many other '*Pseudomonas*' isolates were later reclassified as *Burkholderia*, *Comamonas*, *Stenotrophomonas*, and other genera^{79, 54}. This includes almost all '*Pseudomonas*' isolates that degrade methane and are now called *Methylobacterium*. Another example is '*Pseudomonas butanovora*', which contains a butane monooxygenase similar to soluble methane monooxygenases⁹⁹, and is now thought to belong to the *Thauera* genus. A recent investigation of 128 valid and invalid *Pseudomonas* isolates showed that only 57 belonged to the genus *Pseudomonas* (*sensu stricto*)³.

It is important to note that virtually all alkane-degrading pseudomonads were obtained by the *a posteriori* identification of isolates selected for the ability to utilize alkanes, not by screening available strains of the different *Pseudomonas* species for the ability to grow on alkanes. It should also be noted that the frequency and ease with which alkane-degrading pseudomonads were isolated tells little about the total microbial population involved in the oxidation of alkanes under natural conditions because the enrichment methods may well have had a bias toward the isolation of pseudomonads. Nevertheless, several surveys suggest that pseudomonads

dominate in certain environments (e.g., a contaminated aquifer⁸⁷), although in other cases the Gram-positives were more numerous⁶²

3. ENZYMOLOGY OF ALKANE HYDROXYLATION

In spite of the great diversity of Gram-negative and Gram-positive alkane-degrading organisms, almost all research on the enzymology of bacterial alkane oxidation has been carried out with *P. putida* GPo1 (*P. oleovorans* TF4-1L) and to a lesser extent with *P. aeruginosa* strains Sol 20⁶ and 196 Aa (see ref. [132]), all three containing virtually identical enzyme systems¹²⁴.

Coon and coworkers were the first to report on cell-free enzyme preparations that hydroxylated radioactively labeled *n*-octane to octanoic acid, with octanol and octaldehyde as intermediate oxidation products⁸. The enzyme system responsible for the first oxidation step was originally named ω -hydroxylase based on the ω -oxidation of fatty acids⁶¹, but in later studies the designation 'alkane hydroxylase system' was used, since the enzyme system is believed to have evolved for the utilization of *n*-alkanes as carbon- and energy sources⁷⁴.

Biochemical characterization of the alkane hydroxylase system showed that it consists of three components; a particulate hydroxylase; and two soluble proteins, which act as electron carriers between NADH and the hydroxylase⁸⁴ (Figure 1). The alkane hydroxylase is an integral membrane protein that requires phospholipids and iron for activity, and is inhibited by cyanide⁸⁸. Its primary sequence contains six hydrophobic segments that were shown to span the cytoplasmic membrane as α -helices, based on topology studies employing gene fusions with alkaline phosphatase and β -galactosidase¹²². Four highly conserved sequence motifs contain histidines that are essential for catalytic activity, and are conserved in alkane hydroxylase and xylene monooxygenase sequences as well as in the much more distantly related desaturases^{95, 101, 135}. The conserved histidines probably form the nitrogen-rich coordination sphere for two iron atoms, as shown by Moessbauer spectroscopy⁹⁴.

The two electron transfer proteins that supply electrons for the monooxygenation reaction are rubredoxin and rubredoxin reductase⁸⁴. The reductase is a flavoprotein that transfers electrons from NADH to rubredoxin^{27, 63}. The latter belongs to a family of small electron-transfer proteins containing an iron coordinated by four cysteines⁶⁶. The rubredoxin of *P. putida* GPo1 is unusual in that it consists of two rubredoxin-domains connected by a linker^{57, 65}. Most other rubredoxins involved in alkane hydroxylation (including those in *P. aeruginosa* and *P. fluorescens*) are 'typical' single-domain rubredoxins¹²⁰. Electron transfer between rubredoxin reductase and

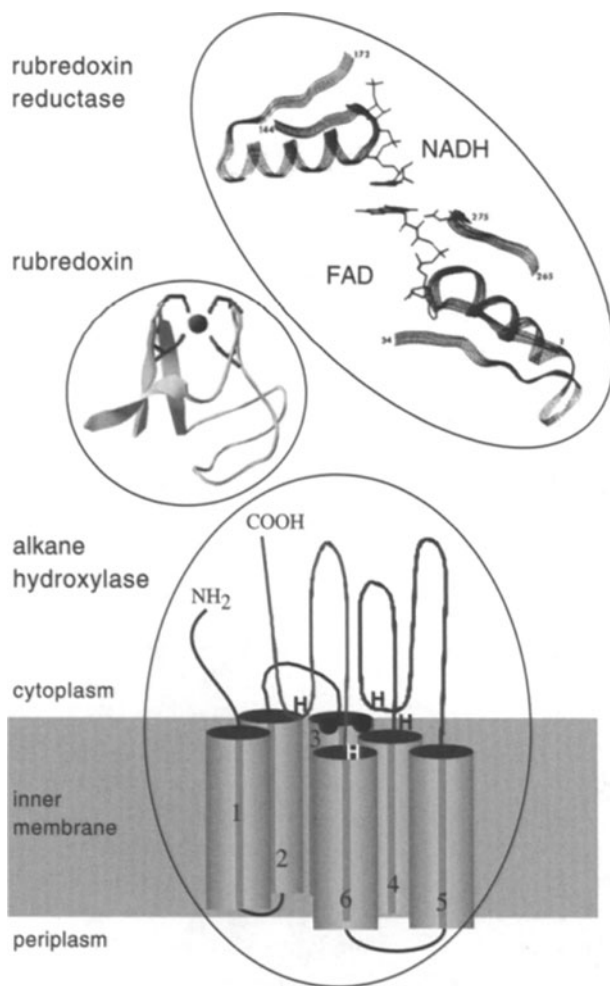


Figure 1. Structure of the membrane-bound alkane hydroxylase system of *P. putida* GPo1. Membrane-bound alkane hydroxylases possess six transmembrane helices¹²², and four conserved histidine-rich motifs (H)^{95, 101, 135}. The two iron atoms in the active site are marked (●). Rubredoxin contains an iron atom marked (●) that is liganded by four cysteines. Several strains contain more than one rubredoxin, or larger proteins consisting of N-terminal and C-terminal rubredoxin domains connected by a linker¹²⁰. Rubredoxin reductase is an FAD-containing NADH-dependent reductase²⁷.

rubredoxin has been studied with isolated and purified proteins⁶³. However, transfer of electrons from rubredoxin to the alkane hydroxylase has not yet been investigated in detail. In vitro, rubredoxin and rubredoxin reductase can be replaced by spinach ferredoxin and ferredoxin reductase¹⁰. The *P. putida*

GPo1 rubredoxin and rubredoxin reductase can be replaced *in vivo* by homologs from Gram-negative as well as Gram-positive alkane-degrading strains¹²⁰.

4. MOLECULAR GENETICS OF ALKANE DEGRADATION BY *PSEUDOMONAS PUTIDA*

In 1973, the *P. putida* enzymes responsible for the oxidation of *n*-octane were shown to be encoded on a large transmissible plasmid named OCT²⁰, and ensuing classical genetics studies led to the identification of two genetic loci encoding the three components of the alkane hydroxylase system, a regulatory protein, and enzymes involved in further degradation steps³¹. The two gene regions containing these loci were subsequently cloned, sequenced, and analyzed for transcripts and translation products^{26, 28, 29, 57, 58, 76, 77, 117}. More recently, a 10 kilobase region separating the two *alk* gene clusters, and other flanking regions, were sequenced and analyzed¹²¹ (Figure 2).

The first *alk* gene cluster was shown to contain the *alkBFGHJKL* genes. These genes constitute an operon²⁹ that encodes the alkane hydroxylase (AlkB), rubredoxin (AlkG), an aldehyde dehydrogenase (AlkH), an alcohol dehydrogenase (AlkJ), an acyl-CoA synthetase (AlkK), and an outer membrane protein (AlkL)^{57, 58, 117}. The *alkF* gene encodes a second rubredoxin that has not been detected in alkane-grown cells, is not essential, and not active in alkane hydroxylation^{57, 120}. The third component of the alkane hydroxylase system, rubredoxin reductase (AlkT), is encoded immediately downstream of the regulator of *alk* gene expression AlkS in the second *alk* gene cluster^{26, 27, 83}.

In another *P. putida* isolate named P1, a very similar gene arrangement was found, except that the *alkST* cluster is located upstream of the *alkBFGHJKL* operon (Figure 2). In both *P. putida* isolates, DNA segments flanking the *alk* genes consist of a mosaic of complete and incomplete insertion sequences. In fact, two almost identical insertion sequences flank the *alk* genes of *P. putida* P1. The combination of both insertion sequences (including the *alk* genes) is in turn flanked by a 4 bp direct repeat (CGTA), while the entire cassette appears to have interrupted another insertion sequence, all features that characterize Class I transposons (Tn*Ppu-alkI*)¹²¹.

A gene located downstream of *alkL* in GPo1 (*alkN*) encodes a homolog of methyl-accepting chemotaxis proteins (quite closely related to the chemotaxis protein for naphthalene⁴¹). The *alkN* promoter region was found to be similar to the *alkB*-promoter and contains an AlkS-binding site, suggestive of a role in chemotaxis toward alkanes. However, direct evidence could not be obtained. In *P. putida* P1, the *alkN* gene is located immediately downstream of *alkL* as an apparent additional cistron in the *alkBFGHJKL* operon. However, it is truncated by one of the flanking insertion sequences. Comparison of the

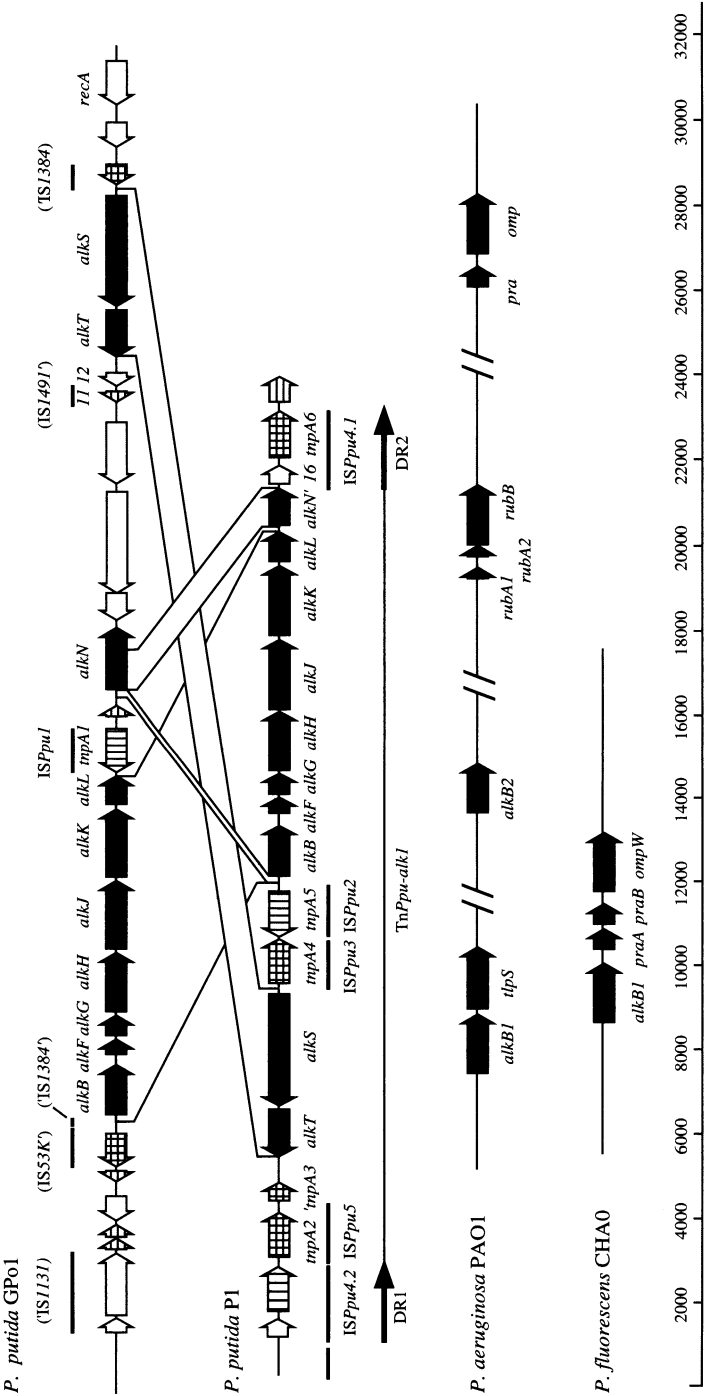


Figure 2. Sequence analysis of the *P. putida* GPo1 and P1 *alk* genes and flanking DNA (adapted from [121]), and organization of *alk* genes in *P. aeruginosa* PAO1 and *P. fluorescens* CHA0. The scale is in bases. Arrows represent potential coding regions. Checked arrows: open-reading-frame interrupted by frame shift or stops. Black arrows represent genes involved, or presumably involved, in alkane degradation, and vertically hatched arrows represent complete or partial transposase genes. Black bars correspond to (incomplete) insertion sequences¹²¹. Lines linking the *P. putida* GPo1 and P1 DNA segments indicate homologous regions. DR: direct repeats formed by *ISPpu4.1* and *ISPpu4.2*.

alkN gene region in both *P. putida* strains suggests that in *P. putida* GPo1, an insertion sequence has interrupted a previously existing *alkBFGHJKLN* operon exactly between *alkL* and *alkN*, and that the promoter-less *alkN* gene subsequently acquired an *alkB* promoter¹²¹.

5. ALKANE DEGRADATION BY OTHER PSEUDOMONADS

As mentioned above, Southern blots and partial sequencing of the *alk* genes showed that four *P. aeruginosa* isolates contain alkane hydroxylases that are virtually identical to the *P. putida* GPo1 enzyme system¹²⁴, and several additional *Pseudomonas* isolates were later found to contain (almost) identical genes as well⁹² (van Beilen *et al.*, unpublished). In general, AlkB homologs that are closely related to the *P. putida* GPo1 enzyme are found frequently but appear to be present in Gram-negative strains only (probably mainly pseudomonads)¹³³. As the *P. putida* GPo1 and P1 *alk* genes are located on the transmissible OCT-plasmid or on a Class 1 transposon, respectively¹²¹, and the two available *P. putida* genome sequences (KT2440 & PRS1) do not contain *alkB*-homologs, *P. putida* is probably not the original host of this particular *alkB*-homolog. A possible source could be marine γ -Proteobacteria that are closely related to the pseudomonads (genera *Marinobacter*, *Alcanivorax*, and *Oleiphilus*, see below)³.

Several research groups have used the *P. putida* GPo1 *alkB* gene⁵⁸ as a gene probe in ecological studies. While in some studies *alkB* homologs were detected in 10–40% of the bacterial population^{105, 109, 110}, other groups did not detect *alkB* homologs at all^{43, 134}. However, only closely related *alkB*-homologs could be detected in these studies.

Unlike the four *P. aeruginosa* isolates Sol 20, 196Aa, KSLA 473, and LA-P-6 that are able to grow on medium-chain length (C5 to C12) as well as long-chain length alkanes (C13 to well over C16), most *P. aeruginosa* isolates (e.g., those obtained from clinical settings) only grow on alkanes longer than C12. These isolates lack close homologs of the *P. putida* GPo1 *alk* genes^{124, 9}, but possess two alkane hydroxylase homologs that have about 35% amino acid sequence identity to the *P. putida* GPo1 alkane hydroxylase^{9, 101, 112}. These long-chain alkane hydroxylases have not been characterized biochemically. However, both alkane hydroxylase genes were shown to encode functional enzymes that oxidize C12–C16 alkanes by heterologous expression in recombinant strains that lack a functional alkane hydroxylase but express all other enzymes and proteins necessary for growth on alkanes. The *P. aeruginosa* PAO1 genome also encodes two rubredoxins and a rubredoxin reductase,

which were shown to be functional in alkane hydroxylation by heterologous expression in similar recombinant strains (Figure 2)^{100, 103}.

The biocontrol strain *P. fluorescens* CHA0 grows on C10–C24 alkanes¹⁰⁰, and was found to contain an alkane hydroxylase that had 50% amino acid sequence identity to the *P. putida* GPo1 enzyme¹⁰¹. Two of the three (unfinished) *P. fluorescens* genome sequences contain a similar gene (strains Pf-5 and SBW25). Deletion of this gene in CHA0 resulted in a more limited growth spectrum: alkanes ranging from C10 to C16 were no longer oxidized. However, longer alkanes were still oxidized, indicating that the strain contains a second alkane hydroxylase¹⁰⁰. The knockout strain *P. fluorescens* KOB2Δ1 proved to be a good host for the expression of alkane hydroxylases from Gram-negative as well as Gram-positive strains¹⁰⁰. In addition, it was possible to use the knockout mutant for the selection of substrate range mutants of the *P. putida* GPo1 alkane hydroxylase: a mutation of W55 to serine or cysteine extended the range of alkanes that can be oxidized by this enzyme from C5–C13 to at least C5–C16¹²³.

Many other *Pseudomonas* strains that degrade alkanes have been described. However, in most cases, further data are not available, and the identity of the isolates has not been confirmed. An exception is the new *Pseudomonas* species named *Pseudomonas indica*⁸¹, which was recently shown to contain a novel alkane hydroxylase system. It has no detectable homology to other oxygenases, but the enzyme has not yet been characterized further⁷⁸.

The closest relatives of the true pseudomonads are marine bacteria belonging to the genera *Marinobacter*, *Alcanivorax*, and *Oleiphilus*³. Interestingly, these bacteria contain *alk* genes that are very closely related to *P. putida* and *P. aeruginosa* *alk* genes. *Alcanivorax borkumensis* contains two alkane hydroxylases. The first shows 75% amino acid sequence identity to the *P. putida* GPo1 alkane hydroxylase, and thus is the closest relative of the GPo1 alkane hydroxylase except for other *P. putida* sequences¹⁰⁰; the second shows 60% sequence identity to the two *P. aeruginosa* alkane hydroxylases, and is also their closest relative apart from other *P. aeruginosa* sequences (van Beilen, unpublished data). Similarly, *Oleiphilus messinensis* also contains a close homolog of the *P. putida* GPo1 alkane hydroxylase gene³⁹.

Many Gram-positive and Gram-negative alkane-degraders, especially those that grow on alkanes longer than C10, contain homologs of the *Pseudomonas* membrane-bound alkane hydroxylases that could be functionally expressed in the *P. fluorescens* *alkB1* knockout strain KOB2Δ1. In a tree of identity, AlkB-homologs obtained from pseudomonads show much greater sequence diversity than any other group of AlkB-homologs, such as those from the *Acinetobacter* strains, or the CNM-*Rhodococcus* group. This strongly suggests that horizontal gene transfer of alkane hydroxylase genes has occurred frequently, especially in the pseudomonads^{100, 119}.

6. REGULATION OF ALKANE DEGRADATION

Our current knowledge of the regulation of alkane oxidation in *P. putida* GPo1 is summarized in Figure 3. A central regulatory element is the *PalkB* promoter, which controls the *alkBFGHJKL* operon⁵⁸. Expression from this promoter requires the presence of the transcriptional activator AlkS, and an inducer such as *n*-octane⁸³. Many compounds other than *n*-alkanes can bind to AlkS and induce expression of the *alk* genes, as was shown first for *P. aeruginosa* KSLA 473¹²⁹, and subsequently for *P. putida* GPo1^{42, 111, 136}. One of these alternative inducers, dicyclopropylketone (DCPK), has been used in many studies as it is non-metabolizable, less volatile than *n*-octane, and as efficient an inducer as *n*-octane⁴². The alkane- and DCPK-inducible promoter *PalkB* has since been exploited for the construction of expression systems for *Escherichia coli* and *Pseudomonas*^{82, 83, 100, 102}, and for the construction of a whole-cell bioluminescent sensor for bioavailable alkanes in groundwater samples¹¹¹.

AlkS itself is encoded by the *alkST* gene cluster, which is controlled by two promoters; *PalkS1* and *PalkS2*. *PalkS1* is σ^S -RNA polymerase-dependent and is essentially silent during exponential growth on a carbon source other than alkanes. In the stationary phase, transcription of the *alkS* gene from *PalkS1* increases^{17, 18}. AlkS has a binding-site that overlaps with *PalkS1*, and therefore acts as a repressor of *PalkS1*. However, in the presence of alkanes, AlkS bound to *PalkS1* can activate expression from *PalkS2*, which is located 38 nucleotides downstream of *PalkS1*, resulting in high expression of the *alkST*-genes¹⁷. The increased amount of AlkS due to this positive feedback mechanism then results in expression of the *alkBFGHJKL* operon from *PalkB*⁵⁸. The binding site of AlkS in *PalkS1* and *PalkB* was shown to be an 18- or 20-nucleotide palindromic sequence by in vivo competition experiments¹²¹.

A further level of control of *alk* gene expression is catabolite repression due to the presence of other carbon-sources^{106, 129, 137}. Luria–Bertani broth caused a more than 50-fold repression of *alk* gene expression in the exponential phase; lactate, glucose, and succinate gave a 3–4-fold reduction, while citrate did not affect *alk* gene repression. Catabolite repression did not occur when the *alk* genes were expressed in *E. coli*¹⁰⁶. The *P. putida* *crc* gene (encoding the catabolite repression control protein) was shown to have an important role in the repression caused by Luria–Bertani broth, but not in that caused by organic acids¹³⁸. A mutation in the major terminal oxidase (cytochrome *o* ubiquinol oxidase) relieved catabolic repression by Luria–Bertani broth as well as organic acids²⁵. Together these levels of *alk* gene expression control are summarized as ‘physiological control’ in Figure 3. For a more general discussion of catabolite repression and physiological control in the pseudomonads, please consult Chapter 13 by F. Rojo and M. A. Dinamarca, Volume 2.

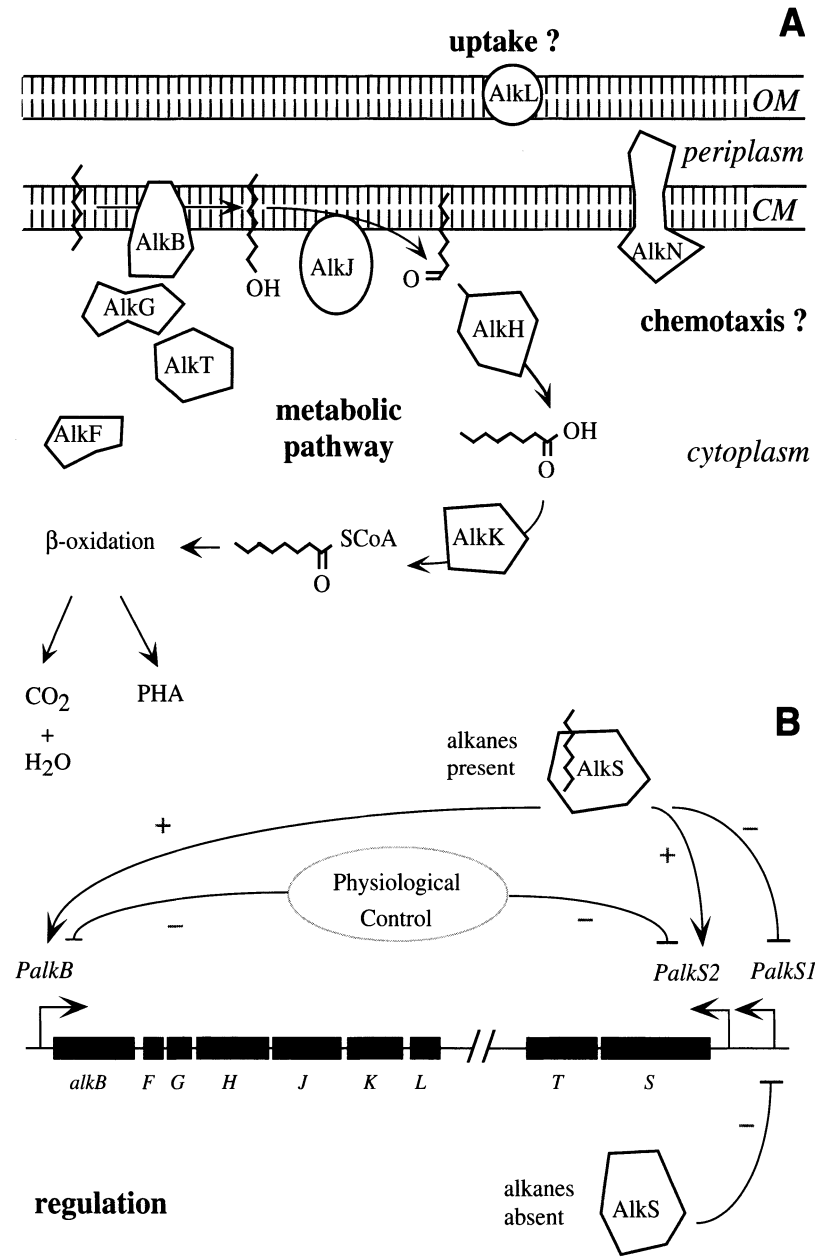


Figure 3. Alkane degradation by *P. putida* GPo1 (adapted from refs [25], [121]). Panel A shows the metabolic pathway of alkane degradation, and the role and cellular localization of Alk proteins. Panel B shows the general structure of the *alk* gene loci, and the regulation of the *alk* genes. For details, see sections on molecular genetics and regulation of alkane degradation.

In *P. putida* GPo1, the two electron transfer components of the alkane hydroxylase system, rubredoxin and rubredoxin reductase, are co-regulated with the alkane hydroxylase¹⁰⁷. However, in *P. aeruginosa* RR1, which contains the same two alkane hydroxylases as *P. aeruginosa* PAO1, an entirely different regulation was observed. Whereas the two alkane hydroxylases were induced by alkanes, and were expressed differentially, depending on the growth phase⁶⁸, rubredoxin and rubredoxin reductase were expressed constitutively. In addition, the two alkane hydroxylase genes in *P. aeruginosa* PAO1 (and probably also in RR1) are not in close proximity to the genes coding for rubredoxin and rubredoxin reductase (*rubA1A2B*) on the genome of *P. aeruginosa* PAO1¹¹². This gene organization and regulation most closely resembles that of *Acinetobacter* spp. ADP1 and M1. In these strains, the *rubAB* operons are also constitutively expressed, in contrast to the alkane hydroxylases, which are induced by alkanes^{38, 86, 114}.

7. DOWNSTREAM METABOLISM

The product of the alkane hydroxylase reaction is usually a 1-alkanol. Most pseudomonads are able to grow on these compounds, even if they do not grow on alkanes; they possess chromosome-encoded alcohol and aldehyde dehydrogenases that convert the 1-alkanol to fatty acids. These are further degraded by β -oxidation to acetyl-CoA, which enters the central metabolism (Figure 3). Under nitrogen or phosphorous limitation 3-hydroxy fatty acid, an intermediate of the β -oxidation cycle can be converted to the carbon-storage material poly-(3-hydroxyalkanoate). If the limitation is relieved the polymer serves as a carbon source.

Several studies describe alcohol and aldehyde dehydrogenases in the isolates *P. aeruginosa* 196 Aa (summarized in ref. [132]), *P. aeruginosa* KSLA 473 (summarized in ref. [128]), *P. aeruginosa* Sol 20^{7, 45}, and *P. putida* GPo1^{11, 57, 117}. Their results can be summarized as follows: *Pseudomonas* strains contain several alcohol dehydrogenases that are constitutively expressed and use NADP⁺ or NAD⁺ as cofactor. These enzymes are most likely chromosome-encoded. Alkane-grown cells express an additional membrane-bound alcohol dehydrogenase, which transfers electrons to artificial electron acceptors such as phenazine methosulfate or ferricyanide^{7, 11, 128, 132}, and indirectly to oxygen if the enzyme is bound to the cytoplasmic membrane¹¹⁷. In *P. putida* GPo1, the gene encoding this enzyme (*alkJ*) was found to be linked to genes encoding the alkane hydroxylase system⁷⁷, which was confirmed by molecular cloning²⁸, sequencing and further enzyme assays¹¹⁷. The membrane-bound soluble-cofactor independent alcohol dehydrogenase

AlkJ belongs to a family of proteins named GMC-oxidoreductases with diverse functions such as methanol and glucose oxidase and choline dehydrogenase¹⁹.

Two NAD⁺ and one NADP⁺-dependent aldehyde dehydrogenases were purified from *P. aeruginosa* 196 Aa, two of which were expressed constitutively. The third (NAD⁺-dependent) aldehyde dehydrogenase was only found in alkane-grown cells¹³², and probably corresponds to AlkH of *P. putida* GPo1⁵⁷. An alkane- or aldehyde-inducible aldehyde dehydrogenase was also found in *P. aeruginosa* Sol 20. It showed activity primarily toward aliphatic aldehydes (heptaldehyde or octaldehyde were the preferred substrates)⁴⁵.

The fatty acids resulting from the sequential action of alcohol and aldehyde dehydrogenase could in principle be activated to acyl-CoA by chromosome-encoded acyl-CoA synthetases. However, analysis of the *P. putida* GPo1 *alkBFGHJKL* operon showed that *alkK* encodes an additional acyl-CoA synthetase (AlkK) that complemented an *E. coli fadD* (acyl-CoA synthetase) mutation¹¹⁷. Its co-induction with the other Alk-proteins may prevent the accumulation of fatty acids. Alternatively, AlkK has a different substrate range than the chromosome-encoded enzyme(s), and is more active toward C5–C12 fatty acids.

8. ALKANE UPTAKE AND SOLUBILIZATION

Generally, alkane uptake is supposed to take place by three different mechanisms, (a) by direct cell–droplet interaction, (b) via uptake of alkanes dissolved in the aqueous phase, or (c) by interaction of cells with solubilized or emulsified hydrocarbon micelles or microdroplets⁴⁰. *Pseudomonas* isolates typically do not use the first option¹³. In the case of short alkanes, the solubility is high enough to allow uptake from dissolved alkanes (for *n*-octane the solubility is 5 μ M; shorter alkanes are more soluble)¹⁶. These short alkanes probably reach the alkane hydroxylase in the cytoplasmic membrane by passive diffusion and partitioning into the cytoplasmic membrane, not by an active uptake system. This is supported by the observation that recombinant *E. coli* strains containing the *P. putida alk* genes grow well on alkanes²⁸, even though the wild-type *E. coli* does not possess pathways for the uptake and degradation of hydrocarbons or the production of biosurfactants.

AlkL, one of the proteins encoded by the *P. putida* GPo1 *alkBFGHJKL* operon, may serve as an alkane porin, as it is located in the outer membrane, and shows homology to an outer-membrane protein possibly involved in the uptake of naphthalene¹¹⁷. However, deletion of the *alkL* gene did not affect the ability of *Pseudomonas* or *E. coli* recombinants to grow on C5–C12 alkanes or to produce 1-alkanols from alkanes in hosts lacking an alcohol dehydrogenase¹¹⁷. The upper operon of the xylene degradation pathway also encodes an outer membrane protein that was shown to be involved in xylene uptake,

and has homology to FadL (fatty acid uptake) and a range of outer membrane proteins encoded by catabolic operons⁵¹.

Longer alkanes are not soluble enough to support growth from dissolved substrate, and in many cases growth on such hydrocarbons is associated with the production of surface-active compounds. In *P. aeruginosa*, an exported protein named Pra or Protein Activator solubilizes alkanes and thereby facilitates alkane uptake by the cell^{44, 46}. Two closely related proteins were found in *P. fluorescens* CHA0, where they are encoded immediately downstream of the alkane hydroxylase gene (Figure 2)¹⁰⁰. Interestingly, an outer membrane-protein with homology to fatty acid porins is encoded immediately downstream of the *P. aeruginosa* as well as the *P. fluorescens pra* genes, and thus could be an alkane-specific porin (Figure 2).

Other factors involved in long-chain alkane degradation are rhamnolipids. Several *P. aeruginosa* mutants unable to grow on alkanes were found to be deficient in the production of rhamnolipids, while growth on alkanes could be restored by the addition of small amounts of rhamnolipids^{49, 56}. In a recent study, it was demonstrated that rhamnolipids do not promote attachment of *P. aeruginosa* to alkane droplets, but rather facilitate uptake of solubilized or emulsified substrate. The uptake of hydrophobic dyes stimulated by rhamnolipids seems to be an energy dependent process⁷⁵, suggesting that this could be true for long-chain alkane uptake as well.

9. PHYSIOLOGY OF ALKANE-DEGRADING PSEUDOMONADS

Growth on hydrophobic compounds such as alkanes can have profound effects on the physiology of a bacterium⁹⁸, and bacteria have developed elaborate mechanisms to counter the effects of solvents on the cell membrane⁸⁵ (see also Volume 1, Chapter 20 by J. J. Rodríguez-Herva and Llamas; Volume 1, Chapter 21 by K. Poole; and Volume 2, Chapter 17 by Segura *et al.*). The specific effects of alkanes on the physiology of alkane-oxidizing *Pseudomonas* strains have been reviewed before¹²⁵. Cultivation of *P. putida* GPo1 in the presence of a bulk second liquid phase consisting of octane results in a number of characteristic changes. These include an increase of the mean acyl-chain length of the membrane phospholipid fatty acids in response to the expression of the integral membrane alkane hydroxylase. A second effect is the conversion of *cis*- to *trans*-unsaturated fatty acids in response to the production of 1-octanol by the alkane hydroxylase²¹. These changes in the membrane lipid fatty acid composition lead to a decrease in membrane lipid fluidity, as shown by differential scanning calorimetry²².

Although *P. putida* GPo1 grows well in the presence of a bulk *n*-octane phase, cells are still damaged, resulting in a cell lysis rate of up to 0.10 hr^{-1} in a continuous culture at $D = 0.21 \text{ hr}^{-1}$. These rates could be lowered by 50–70% by diluting *n*-octane in non-metabolizable long-chain hydrocarbon solvents⁹¹. The cell lysis is probably related to the formation of large amounts of biosurfactant by *P. putida*. This biosurfactant has been identified as consisting mainly of lipopolysaccharide, but also contain a significant amount of protein and lipids. The composition is most likely the result of a mix of specific cellular surfactant excretion and cell lysis caused by toxic effects of organic solvents⁹⁰.

10. BIOCATALYSIS BY ALKANE-DEGRADING PSEUDOMONADS

Two *Pseudomonas* isolates: *P. putida* GPo1 (*P. oleovorans* TF4-1L) and *P. aeruginosa* KSLA 473, which were later shown to contain almost identical alkane hydroxylase systems, are the most thoroughly studied pseudomonads with regard to applications in the production of fine-chemicals. In a series of publications, *P. aeruginosa* KSLA 473 was shown to hydroxylate a wide range of linear and branched alkanes, alicyclic compounds, and alkylbenzenes¹³¹. The same strain was earlier shown to catalyze the epoxidation of terminal olefins^{126, 47}.

After the initial observations by Coon and coworkers that the *P. putida* GPo1 alkane hydroxylase system catalyzes the ω -hydroxylation of fatty acids and the terminal hydroxylation of alkanes⁷², the *P. putida* GPo1 alkane hydroxylase system was also shown to catalyze epoxidation reactions⁶⁹. This useful reaction was subsequently studied and exploited by Abbott and coworkers at Esso Research (now Exxon), who showed that 1-alkenes ranging from C6 to C12 could be epoxidated¹, and that 1,7-octadiene is epoxidated at both ends⁷⁰. Production of 1,2-epoxyoctane in a bioreactor was optimized by repeatedly transferring a 1-octene layer containing 1,2-epoxyoctane to fresh growing cultures of *P. oleovorans* GPo1²⁴. Gist-brocades (now DSM) and Shell used several bacteria including *P. putida* GPo1, *P. aeruginosa* Sol 20, *P. aeruginosa* 196 Aa, for the stereospecific epoxidation of 4-(2-methoxyethyl)phenylallyl ether, an intermediate in the production of *S*-(-)- β -blockers⁵⁰. A series of allyl benzyl ethers and other allylic compounds also yielded epoxides with variable enantiomeric excess³⁶.

The alkane hydroxylase system catalyzes several reactions besides hydroxylation and epoxidation, such as the oxidation of terminal alcohols to the corresponding aldehydes⁷¹; the oxidation of 1-octene to 1-octanal as well as 1,2-epoxyoctane⁵³; sulfoxidation of thioethers and the demethylation

of branched methyl ethers⁵² (Figure 4, reactions 3, 5, 6, 8). Compounds 14 and 15 (Figure 4) are diagnostic substrates that undergo structural changes depending on the nature of the reaction intermediates. In the case of reaction 13, 1-phenyl-2-buten-1-ol was the only product, which indicates that the reaction proceeds through a nonconcerted radical process³⁶, but the product does not rule out a cationic pathway. Norcarnane hydroxylation (reaction 15), however, only produces reactants characteristic of a radical process, and does not yield cyclohept-3-en-1-ol, which would have been indicative of a cationic pathway⁵.

In most substrate range studies, cell extracts of (partially) purified enzyme preparations were used. However, this is not practical for the production of fine-chemicals. Whole cells of the wild-type strain can be used for the production of epoxides, as these compounds appear to be dead-end metabolites in the alkane-degrading *Pseudomonas* strains discussed here. However, aliphatic or aromatic alcohols produced by *Pseudomonas* strains expressing an alkane hydroxylase are rapidly metabolized due to the presence of active alcohol and aldehyde dehydrogenases in *P. putida* GPo1 (see above). Therefore, Bosetti *et al.* (1992), introduced plasmid pGEc41, which encodes the alkane hydroxylase system but lacks the alcohol dehydrogenase gene *alkJ* that is part of the *alk*-system²⁹, into the *P. putida* strain PpS81, which lacks one of the chromosome-encoded medium chain-length alcohol dehydrogenase activities (AlcA)⁴². This recombinant (PpS8141) was then used to synthesize and accumulate 1-alkanols from linear alkanes¹².

Using the same recombinant *P. putida* strain, the *in vivo* conversion rates of a large set of linear, branched and cyclic alkanes, and alkylbenzenes, was investigated¹¹⁸. These results were quite similar to those obtained earlier for *P. aeruginosa* KSLA 473¹³¹, in retrospect not surprising as the alkane hydroxylase systems in these strains are almost identical¹²⁴. In these studies, hydrocarbons were not hydroxylated when a tertiary carbon was present. Substituted cyclic alkanes were oxidized with high stereoselectivity at the 4-*trans* position of the ring relative to the substituents, but not at the methyl- or ethyl substituents themselves (Figure 4, reaction 7). Several alkylbenzenes such as ethylbenzene (reaction 12) and its 3- and 4-substituted derivatives were oxidized with rates close to or superior to the rate for *n*-nonane¹¹⁸. The regioselective oxidation of ethyl-substituted aromatic compounds has been exploited in industry to produce hydroxyethyl-substituted 5- or 6-membered heterocyclic compounds, such as 5-hydroxyethyl-2-methylpyrimidine (Figure 4, reaction 11)⁵⁵.

The combination of a wide substrate range with high regio- and stereoselectivity makes the *Pseudomonas* alkane hydroxylase systems useful for the production of fine-chemicals such as fatty acids, alcohols, epoxides, and sulfoxides, as will be discussed in more detail in Volume 3 (section entitled Catabolism and Biotransformations).

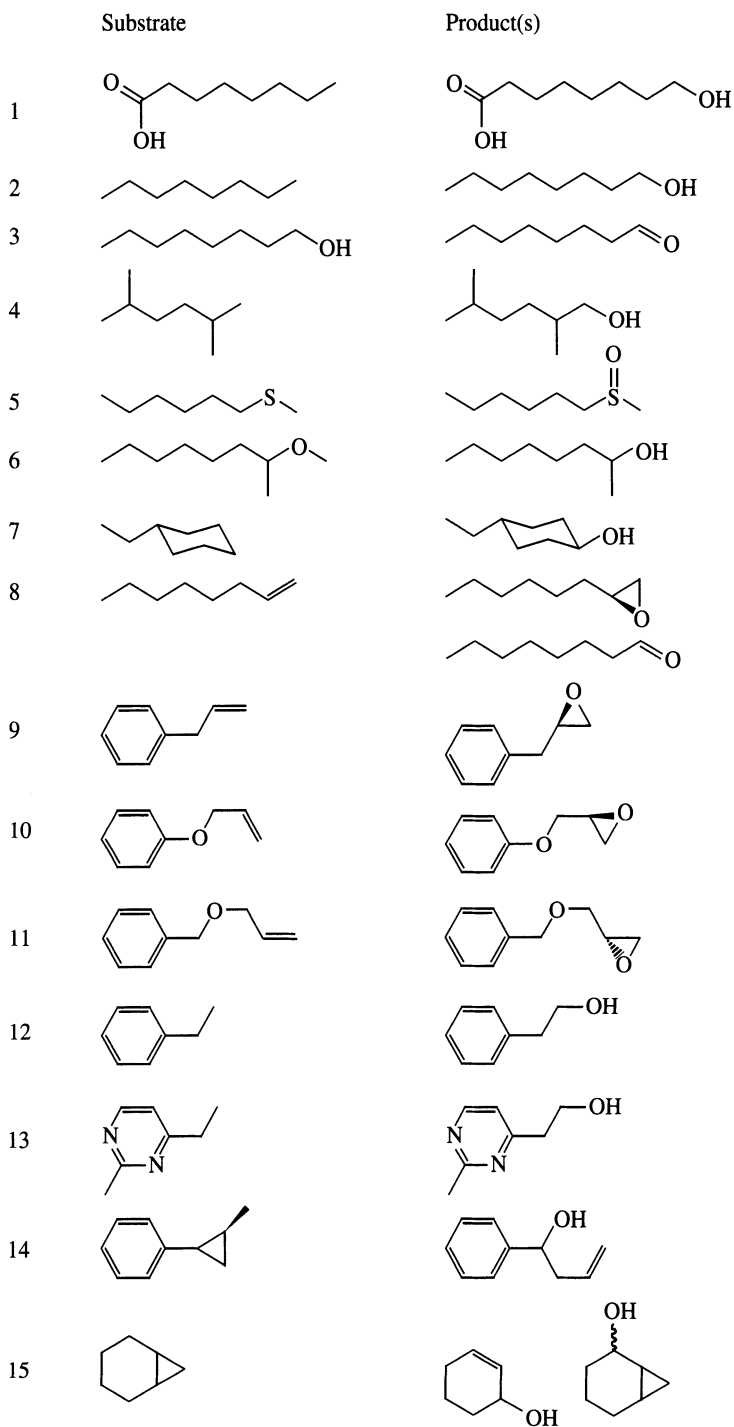


Figure 4. Oxidation reactions catalyzed by the *P. putida* alkane hydroxylase system. Reaction 1: ref. [8]; reaction 2: ref. [61]; reaction 3: ref. [71]; reactions 4, 7, 12: ref. [118]; reactions 5, 6: ref. [52]; reaction 8: ref. [69]; reaction 9: ref. [23]; reaction 10: ref. [50]; reactions 11, 14: ref. [36]; reaction 13: ref. [55]; reaction 15: ref. [5].

11. ALTERNATIVE PATHWAYS FOR THE BIODEGRADATION OF LINEAR, BRANCHED, AND CYCLIC ALKANES

Subterminal oxidation of alkanes has been detected for several organisms, including pseudomonads^{14, 35}. The resulting secondary alcohols are converted to the corresponding ketones^{33, 35}, which can be oxidized to an ester by Baeyer–Villiger monooxygenases^{15, 32, 34}. The esters in turn are hydrolyzed by an esterase to an alcohol and a fatty acid^{96, 97}. The Baeyer–Villiger monooxygenases and esterases have been characterized to some extent, however, oxygenases responsible for subterminal oxidation of alkanes by pseudomonads have not been identified yet.

Branched alkanes are considered to be more recalcitrant to degradation than linear alkanes because (a) branching inhibits the initial hydroxylation step^{118, 132}, (b) branched compounds do not induce *alk* genes¹¹¹, (c) branching inhibits or blocks the regular β -oxidation pathway, as demonstrated for β -alkyl-branched alkanes⁸⁹. Reports of pseudomonads that grow on branched or cyclic alkanes are scarce. Decane-utilizing mutants of *Pseudomonas citronellolis*, which grows on citronellol and C12–C16 alkanes, were able to grow on 2,6-dimethyloctane³⁰. Cyclohexane-degrading *Pseudomonas* isolates were described already in 1948⁴⁸, and in 1980², but these strains were not studied further. Cyclic and branched alkanes are hydroxylated with relatively high rates by the *P. putida* GPO1 and *P. aeruginosa* KSLA 473 alkane hydroxylases^{118, 131}. However, these strains cannot grow on the oxidation products, probably because they lack appropriate enzymes for the downstream metabolism.

12. CONCLUSIONS AND OUTLOOK

Much of the work on alkane hydroxylation by pseudomonads has focused on a few very closely related enzyme systems, and detailed structural information has not been forthcoming because the monooxygenase component of the alkane hydroxylase system is an integral membrane protein. Now that functional heterologous expression of a series of integral membrane hydroxylases and the selection of substrate range mutants has been achieved, the further functional characterization and modification of the substrate range of these enzymes has become feasible. Alkane hydroxylases in pseudomonads thus provide an important model system for the study of (distantly) related—also integral membrane—enzymes such as alkane hydroxylases in other microorganisms, xylene monooxygenases, desaturases, and enzymes that catalyze decarbonylation or the introduction of keto-groups in carotenes. The *Pseudomonas* enzymes are interesting in their own right as biocatalysts for

the production of a wide range of aliphatic and aromatic alcohols, aldehydes, acids, epoxides, and sulfoxides. The recent description of a new type of oxygenase in *P. indica*, a new *Pseudomonas* species, suggests that there are still novel alkane hydroxylases to be discovered in the pseudomonads.

REFERENCES

1. Abbott, B.J. and Hou, C.T., 1973, Oxidation of 1-alkenes to 1,2-epoxyalkanes by *Pseudomonas oleovorans*. *Appl. Microbiol.*, 26:86–91.
2. Anderson, M.S., Hall, R.A., and Griffin, M., 1980, Microbial metabolism of alicyclic hydrocarbons: Cyclohexane catabolism by a pure strain of *Pseudomonas* sp. *J. Gen. Microbiol.*, 120:89–94.
3. Anzai, Y., Kim, H., Park, J.Y., Wakabayashi, H., and Oyaizu, H., 2000, Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. *Int. J. Syst. Evol. Microbiol.*, 50:1563–1589.
4. Atlas, R.M. and Bartha, R., 1992, Hydrocarbon biodegradation and oil spill bioremediation. *Adv. Microb. Ecol.*, 12:287–338.
5. Austin, R.N., Chang, H.-K., Zylstra, G.J., and Groves, J.T., 2000, The non-heme diiron alkane monooxygenase of *Pseudomonas oleovorans* (AlkB) hydroxylates via a substrate radical intermediate. *J. Am. Chem. Soc.*, 122:11747–11748.
6. Azoulay, E., Davidovics, G., and Chouteau, J., 1963, Isolement et caractérisation des enzymes responsables de l'oxydation des hydrocarbures. *Biochim. Biophys. Acta*, 77:554–567.
7. Azoulay, E. and Heydeman, M.T., 1963, Extraction and properties of alcohol dehydrogenase from *Pseudomonas aeruginosa*. *Biochim. Biophys. Acta*, 73:1–6.
8. Baptist, J.N., Gholson, R.K., and Coon, M.J., 1963, Hydrocarbon oxidation by a bacterial enzyme system: I Products of octane oxidation. *Biochim. Biophys. Acta*, 69:40–47.
9. Belhaj, A., Desnoues, N., and Elmerich, C., 2002, Alkane biodegradation in *Pseudomonas aeruginosa* strains isolated from a polluted zone: Identification of *alkB* and *alkB*-related genes. *Res. Microbiol.*, 153:339–344.
10. Benson, S., Fennewald, M., Shapiro, J., and Huettner, C., 1977, Fractionation of inducible alkane hydroxylase activity in *Pseudomonas putida* and characterization of hydroxylase-negative plasmid mutations. *J. Bacteriol.*, 132:614–621.
11. Benson, S. and Shapiro, J., 1976, Plasmid determined alcohol dehydrogenase activity in alkane-utilizing strain of *Pseudomonas putida*. *J. Bacteriol.*, 126:794–798.
12. Bosetti, A., van Beilen, J.B., Preusting, H., Lageveen, R.G., and Witholt, B., 1992, Production of primary aliphatic alcohols with a recombinant *Pseudomonas* strain, encoding the alkane hydroxylase enzyme system. *Enzyme Microb. Technol.*, 14:702–708.
13. Bouchez-Naitali, M., Rakatozafy, H., Marchal, R., Leveau J.-Y., and Vandecasteele J.-P., 1999, Diversity of bacterial strains degrading hexadecane in relation to the mode of substrate uptake. *J. Appl. Microbiol.*, 86:421–428.
14. Britton, L.N., 1984, Microbial degradation of aliphatic hydrocarbons. In D.T. Gibson (ed.), *Microbial Degradation of Organic Compounds*, pp. 89–129. Marcel Dekker, New York.
15. Britton, L.N. and Markovetz, A.J., 1977, A novel ketone monooxygenase from *Pseudomonas cepacia*. Purification and properties. *J. Biol. Chem.*, 252:8561–8566.
16. Cameotra, S.S. and Singh, H.D., 1990, Uptake of volatile n-alkanes by *Pseudomonas* PG-1. *J. Microb. Biotechnol.*, 5:47–57.

17. Canosa, I., Sanchez-Romero, J.M., Yuste, L., and Rojo, F., 2000, A positive feedback mechanism controls expression of AlkS, the transcriptional regulator of the *Pseudomonas oleovorans* alkane degradation pathway. *Mol. Microbiol.*, 35:791–799.
18. Canosa, I., Yuste, L., and Rojo, F., 1999, Role of the alternative sigma factor σ^S in the expression of the AlkS regulator of the *Pseudomonas oleovorans* alkane degradation pathway. *J. Bacteriol.*, 181:1748–1754.
19. Cavener, D.R., 1992, GMC oxidoreductases: A newly defined family of homologous proteins with diverse catalytic activities. *J. Mol. Biol.*, 223:811–814.
20. Chakrabarty, A.M., Chou, G., and Gunsalus, I.C., 1973, Genetic regulation of octane dissimilation plasmid in *Pseudomonas*. *Proc. Natl. Acad. Sci. USA*, 70:1137–1140.
21. Chen, Q., Janssen, D.B., and Witholt, B., 1995, Growth on octane alters the membrane lipid fatty acids of *Pseudomonas oleovorans* due to the induction of *alkB* and synthesis of octanol. *J. Bacteriol.*, 177:6894–6901.
22. Chen, Q., Nijenhuis, A., Preusting, H., Dolfing, J., Janssen, D.B., and Witholt, B., 1995, Effects of octane on the fatty acid composition and transition temperature of *Pseudomonas oleovorans* membrane lipids during growth in two-liquid-phase continuous cultures. *Enzyme Microb. Technol.*, 17:647–652.
23. de Smet M.-J., Kingma, J., Wynberg, H., and Witholt, B., 1983, *Pseudomonas oleovorans* as a tool in bioconversions of hydrocarbons: Growth, morphology and conversion characteristics in different two-phase systems. *Enzyme Microb. Technol.*, 5:352–360.
24. de Smet M.-J., Wijnberg, H., and Witholt, B., 1981, Synthesis of 1,2-epoxyoctane by *Pseudomonas oleovorans* during growth in a two phase system containing high concentrations of 1-octene. *Appl. Environ. Microbiol.*, 42:811–816.
25. Dinamarca, M.A., Ruiz, M.A., and Rojo, F., 2002, Inactivation of cytochrome o ubiquinol oxidase relieves catabolic repression of the *Pseudomonas putida* GPo1 alkane degradation pathway. *J. Bacteriol.*, 184:3785–3793.
26. Eggink, G., Engel, H., Meijer, W., Otten, J., Kingma, J., and Witholt, B., 1988, Alkane utilization in *Pseudomonas oleovorans*. Structure and function of the regulatory locus *alkR*. *J. Biol. Chem.*, 263:13400–13405.
27. Eggink, G., Engel, H., Vriend, G., Terpstra, P., and Witholt, B., 1990, Rubredoxin reductase of *Pseudomonas oleovorans*. Structural relationship to other flavoprotein oxidoreductases based on one NAD and two FAD fingerprints. *J. Mol. Biol.*, 212:135–142.
28. Eggink, G., Lageveen, R.G., Altenburg, B., and Witholt, B., 1987, Controlled and functional expression of *Pseudomonas oleovorans* alkane utilizing system in *Pseudomonas putida* and *Escherichia coli*. *J. Biol. Chem.*, 262:17712–17718.
29. Eggink, G., van Lelyveld, P.H., Arnberg, A., Arfman, N., Witteveen, C., and Witholt, B., 1987, Structure of the *Pseudomonas putida* *alkBAC* operon. Identification of transcription and translation products. *J. Biol. Chem.*, 262:6400–6406.
30. Fall, R.R., Brown, J.L., and Schaeffer, T.L., 1979, Enzyme recruitment allows the biodegradation of recalcitrant branched hydrocarbons by *Pseudomonas citronellolis*. *Appl. Environ. Microbiol.*, 38:715–722.
31. Fennewald, M., Benson, S., Oppici, M., and Shapiro, J., 1979, Insertion element analysis and mapping of the *Pseudomonas* plasmid *alk* regulon. *J. Bacteriol.*, 139:940–952.
32. Forney, F.W. and Markovetz, A.J., 1968, Oxidative degradation of methyl ketones II. Chemical pathway for degradation of 2-tridecanone by *Pseudomonas multivorans* and *Pseudomonas aeruginosa*. *J. Bacteriol.*, 96:1055–1064.
33. Forney, F.W., and Markovetz, A.J., 1970, Subterminal oxidation of aliphatic hydrocarbons. *J. Bacteriol.*, 102:281–282.
34. Forney, F.W., Markovetz, A.J., and Kallio, R.E., 1967, Bacterial oxidation of 2-tridecanone to 1-undecanol. *J. Bacteriol.*, 93:649–655.

35. Fredricks, K.M., 1967, Products of the oxidation of *n*-decane by *Pseudomonas aeruginosa* and *Mycobacterium rhodochrous*. *Antonie van Leeuwenhoek*, 33:41–48.
36. Fu, H., Newcomb, M., and Wong, C.H., 1991, *Pseudomonas oleovorans* monooxygenase catalyzed asymmetric epoxidation of allyl alcohol derivatives and hydroxylation of a hypersensitive radical probe with the radical ring opening state exceeding the oxygen rebound state. *J. Am. Chem. Soc.*, 113:5878–5880.
37. Fuhs, G.W., 1961, Der mikrobielle Abbau von Kohlenwasserstoffen. *Arch. Mikrobiol.*, 39:374–422.
38. Geißdörfer, W., Kok, R.B., Ratajczak, A., Hellingwerf, K.J., and Hillen, W., 1999, The genes *rubA* and *rubB* for alkane degradation in *Acinetobacter* sp. strain ADP1 are in an operon with *estB*, encoding an esterase, and *oxyR*. *J. Bacteriol.*, 181:4292–4298.
39. Golyshin, P.N., Chernikova, T., Abraham, W.R., Lünsdorf, H., Timmis, K.N., and Yakimov, M.M., 2002, *Oleiphilaceae* fam. nov., to include *Oleiphilus messinensis* gen. nov., sp. nov., a novel marine bacterium that obligately utilizes hydrocarbons. *Int. J. Syst. Evol. Microbiol.*, 52:901–911.
40. Goswami, P. and Singh, H.D., 1991, Different modes of hydrocarbon uptake by two *Pseudomonas* species. *Biotechnol. Bioeng.*, 37:1–11.
41. Grimm, A.C. and Harwood, C.S., 1999, NahY, a catabolic plasmid-encoded receptor required for chemotaxis of *Pseudomonas putida* to the aromatic hydrocarbon naphthalene. *J. Bacteriol.*, 181:3310–3316.
42. Grund A. *et al.*, 1975, Regulation of alkane oxidation in *Pseudomonas putida*. *J. Bacteriol.*, 123:546–556.
43. Guo, C., Sun, W., Harsh, J.B., and Ogram, A., 1997, Hybridisation analysis of microbial DNA from fuel oil-contaminated and noncontaminated soil. *Microb. Ecol.*, 34:178–187.
44. Hardegger, M., Koch, A.K., Ochsner, U.A., Fiechter, A., and Reiser, J., 1994, Cloning and heterologous expression of a gene encoding an alkane-induced extracellular protein involved in alkane assimilation from *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.*, 60:3679–3687.
45. Heydeman, M.T. and Azoulay, E., 1963, Extraction and properties of aldehyde dehydrogenase from *Pseudomonas aeruginosa*. *Biochim. Biophys. Acta*, 77:545–553.
46. Hisatsuka, K., Nakahara, T., and Yamada, K., 1972, Protein-like activator for *n*-alkane oxidation by *Pseudomonas aeruginosa* S7B1. *Agr. Biol. Chem.*, 36:1361–1369.
47. Huybregtse, R. and van der linden, A.C., 1964, Oxidation of α -olefins by *Pseudomonas*—Reactions involving double bond. *Antonie van Leeuwenhoek*, 30:185–196.
48. Imelik, B., 1948, Oxydation du cyclohexane par *Pseudomonas aeruginosa*. *C. R. Hebd. Séances Acad. Sci.*, 226:2082–2083.
49. Itoh, S. and Suzuki, T., 1972, Effect of rhamnolipids on growth of *Pseudomonas aeruginosa* mutant deficient in paraffin-utilizing ability. *Agric. Biol. Chem.*, 36:2233–2235.
50. Johnstone S.L. *et al.*, 1986, Stereoselective synthesis of S(-)- β -blockers via microbially produced epoxide intermediates. In C. Laane, J. Tramper, and M.D. Lilly (eds), *Biocatalysis in Organic Media*, pp. 387–392. Elsevier, Amsterdam.
51. Kasai, Y., Inoue, J., and Harayama, S., 2001, The TOL plasmid pWWO *xylN* gene product from *Pseudomonas putida* is involved in m-xylene uptake. *J. Bacteriol.*, 183:6662–6666.
52. Katopodis, A.G., Smith, H.A., and May, S.W., 1988, New oxyfunctionalization capabilities for ω -hydroxylases: Asymmetric aliphatic sulfoxidation and branched ether demethylation. *J. Am. Chem. Soc.*, 110:897–899.
53. Katopodis, A.G., Wimalasena, K., Lee, J., and May, S.W., 1984, Mechanistic studies on non-heme iron monooxygenase catalysis: Epoxidation, aldehyde formation, and demethylation by the ω -hydroxylation system of *Pseudomonas oleovorans*. *J. Am. Chem. Soc.*, 106:7928–7935.

54. Kersters, K., Ludwig, W., Vancanneyt, M., DeVos, P., Gillis, M., and Schleifer, K.H., 1996, Recent changes in the classification of the pseudomonads: An overview. *Syst. Appl. Microbiol.*, 19:465–477.
55. Kiener, A. and Zimmermann, T., 1992, Microbiological process for terminal hydroxylation of ethyl-groups on aromatic 5- or 6-membered heterocycles. EP 502524, Switzerland.
56. Koch, A.K., Kappeli, O., Fiechter, A., and Reiser, J., 1991, Hydrocarbon assimilation and biosurfactant production in *Pseudomonas aeruginosa* mutants. *J. Bacteriol.*, 173:4212–4219.
57. Kok, M., Oldenhuis, R., van der Linden M.P.G., Meulenberg C.H.C., Kingma, J., and Witholt, B., 1989, The *Pseudomonas oleovorans* *alkBAC* operon encodes two structurally related rubredoxins and an aldehyde dehydrogenase. *J. Biol. Chem.*, 264:5442–5451.
58. Kok M. *et al.*, 1989, The *Pseudomonas oleovorans* alkane hydroxylase gene. Sequence and expression. *J. Biol. Chem.*, 264:5435–5441.
59. Konovaltschikoff-Mazoyer, M. and Senez, J.C., 1956, Degradation bacterienne des hydrocarbures paraffiniques .1. Isolement et caracterisation de souches marines et terrestres appartenant au genre *Pseudomonas*. *Ann. Inst. Pasteur*, 91:60–67.
60. Kusunose, M., Ichihara, K., Kusunose, E., Nozaka, J., and Matsumoto, J., 1967, The possible role of flavin on the hydroxylation of hydrocarbon by bacterial enzyme system. *Agric. Biol. Chem.*, 31:990–992.
61. Kusunose, M., Kusunose, E., and Coon, M.J., 1964, Enzymatic ω -oxidation of fatty acids. I. Products of octanoate, decanoate, and laurate oxidation. *J. Biol. Chem.*, 239:1374–1380.
62. Leahy, J.G., and Colwell, R.R., 1990, Microbial degradation of hydrocarbons in the environment. *Microbiol. Rev.*, 54:305–315.
63. Lee, H.J., Basran, J., and Scrutton, N.S., 1998, Electron transfer from flavin to iron in the *Pseudomonas oleovorans* rubredoxin reductase-rubredoxin electron transfer complex. *Biochemistry*, 37:15513–15522.
64. Lee, M. and Chandler, A.C., 1941, A study of the nature, growth and control of bacteria in cutting compounds. *J. Bacteriol.*, 41:373–386.
65. Lode, E.T. and Coon, M.J., 1971, Enzymatic ω -oxidation. V. Forms of *Pseudomonas oleovorans* rubredoxin containing one or two iron atoms: Structure and function in ω -hydroxylation. *J. Biol. Chem.*, 246:791–802.
66. Lovenberg, W. and Sobel, B.E., 1965, Rubredoxin: A new electron transfer protein from *Clostridium pasteurianum*. *Proc. Natl. Acad. Sci. USA*, 54:193–199.
67. Macham, L.P. and Heydeman, M.T., 1974, *Pseudomonas aeruginosa* mutants defective in heptane oxidation. *J. Gen. Microbiol.*, 85:77–84.
68. Marin, M., Yuste, L., and Rojo, F., 2003, Differential expression of the components of the two alkane hydroxylases from *Pseudomonas aeruginosa*. *J. Bacteriol.*, 185:3232–3237.
69. May, S.W. and Abbott, B.J., 1972, Enzymatic epoxidation. I. Alkane epoxidation by the ω -hydroxylation system of *Pseudomonas oleovorans*. *Biochem. Biophys. Res. Comm.*, 48:1230–1234.
70. May, S.W. and Abbott, B.J., 1973, Enzymatic epoxidation. II. Comparison between the epoxidation and hydroxylation reactions catalyzed by the ω -hydroxylation system of *Pseudomonas oleovorans*. *J. Biol. Chem.*, 248:1725–1730.
71. May, S.W. and Katopodis, A.G., 1986, Oxygenation of alcohol and sulphide substrates by a prototypical non-haem iron monooxygenase: Catalysis and biotechnological potential. *Enzyme Microb. Technol.*, 8:17–21.
72. McKenna, E.J. and Coon, M.J., 1970, Enzymatic ω -oxidation. IV. Purification and properties of the ω -hydroxylase of *Pseudomonas oleovorans*. *J. Biol. Chem.*, 245:3882–3889.
73. National Research Council, 2002, Oil in the sea III: Inputs, fates, and effects. National Academy Press, Washington DC.

74. Nieder, M. and Shapiro, J., 1975, Physiological function of the *Pseudomonas putida* PpG6 *Pseudomonas oleovorans* alkane hydroxylase: Monoterminal oxidation of alkane and fatty acids. *J. Bacteriol.*, 122:93–98.
75. Noordman, W.H. and Janssen, D.B., 2002, Rhamnolipid stimulates uptake of hydrophobic compounds by *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.*, 68:4502–4508.
76. Owen, D.J., 1986, Molecular cloning and characterization of sequences from the regulatory cluster of the *Pseudomonas* plasmid *alk* system. *Mol. Gen. Genet.*, 203:64–72.
77. Owen D.J. *et al.*, 1984, Physical structure, genetic content and expression of the *alkBAC* operon. *Mol. Gen. Genet.*, 197:373–383.
78. Padda R.S. *et al.*, 2001, A novel gene encoding a 54 kDa polypeptide is essential for butane utilization by *Pseudomonas* sp. IMT37. *Microbiology*, 147:2479–2491.
79. Palleroni, N.J., 1984, Genus I. *Pseudomonas* Migula 1894. In N.R. Krieg, J.G. Holt (eds), *Bergey's Manual of Systematic Bacteriology*. Williams & Wilkins, Baltimore
80. Palleroni, N.J., Kunisawa, R., Contopoulou, R., and Douderoff, M., 1973, Nucleic acid homologies in the genus *Pseudomonas*. *Int. J. Syst. Bacteriol.*, 23:333–339.
81. Pandey, K.K., Mayilray, S., and Chakrabarti, T., 2002, *Pseudomonas indica* sp. nov., a novel butane utilizing species. *Int. J. Syst. Evol. Microbiol.*, 52:1559–1567.
82. Panke, S., De Lorenzo, V., Kaiser, A., Witholt, B., and Wubbolts, M.G., 1999, Engineering of a stable whole-cell biocatalyst capable of (*S*)-styrene oxide formation for continuous two-liquid phase applications. *Appl. Environ. Microbiol.*, 65:5619–5623.
83. Panke, S., Meyer, A., Huber, C.M., Witholt, B., and Wubbolts, M.G., 1999, An alkane-responsive expression system for the production of fine chemicals. *Appl. Environ. Microbiol.*, 65:2324–2332.
84. Peterson, J.A., Basu, D., and Coon, M.J., 1966, Enzymatic ω -oxidation. I. Electron carriers in fatty acid and hydrocarbon hydroxylation. *J. Biol. Chem.*, 241:5162–5164.
85. Ramos J.L. *et al.*, 2002, Mechanisms of solvent tolerance in Gram-negative bacteria. *Annu. Rev. Microbiol.*, 56:743–768.
86. Ratajczak, A., Geißdörfer, W., and Hillen, W., 1998, Expression of alkane hydroxylase from *Acinetobacter* sp. strain ADP1 is induced by a broad range of *n*-alkanes and requires the transcriptional activator AlkR. *J. Bacteriol.*, 180:5822–5827.
87. Ridgway, H.F., Safarik, J., Phipps, D., Carl, P., and Clark, D., 1990, Identification and catabolic activity of well-derived gasoline-degrading bacteria from a contaminated aquifer. *Appl. Environ. Microbiol.*, 56:3565–3575.
88. Ruettinger, R.T., Griffith, G.R., and Coon, M.J., 1977, Characterization of the ω -hydroxylase of *Pseudomonas oleovorans* as a non-heme iron protein. *Arch. Biochem. Biophys.*, 183:528–537.
89. Schaeffer, T.L., Cantwell, S.G., Brown, J.L., Watt, D.S., and Fall, R.R., 1979, Microbial growth on hydrocarbons: Terminal branching inhibits biodegradation. *Appl. Environ. Microbiol.*, 38:742–746.
90. Schmid, A., Kollmer, A., and Witholt, B., 1998, Effects of biosurfactant and emulsification on two-liquid phase *Pseudomonas oleovorans* cultures and cell-free emulsions containing *n*-decane. *Enz. Microb. Technol.*, 22:487–493.
91. Schmid, A., Sonnleitner, B., and Witholt, B., 1998, Medium chain length alkane solvent-cell transfer rates in two-liquid phase, *Pseudomonas oleovorans* cultures. *Biotechnol. Bioeng.*, 60:10–23.
92. Schorcht, S., 1998, Mikrobiologische und molekularbiologische Charakterisierung alkanabbauender Bakteriengemeinschaften. Ph.D thesis. Universität Bremen, Bremen.
93. Schwartz, R.D. and McCoy, C.J., 1973, *Pseudomonas oleovorans* hydroxylation-epoxidation system: Additional strain improvements. *Appl. Microbiol.*, 26:217–218.

94. Shanklin, J., Achim, C., Schmidt, H., Fox, B.G., and Münck, E., 1997, Mössbauer studies of alkane ω -hydroxylase: Evidence for a diiron cluster in an integral-membrane protein. *Proc. Natl. Acad. Sci. USA*, 94:2981–2986.
95. Shanklin, J., Whittle, E., and Fox, B.G., 1994, Eight histidine residues are catalytically essential in a membrane-associated iron enzyme, stearyl-CoA desaturase, and are conserved in alkane hydroxylase and xylene monooxygenase. *Biochemistry*, 33:12787–12794.
96. Shum, A.C. and Markovetz, A.J., 1974, Purification and properties of undecyl acetate esterase from *Pseudomonas cepacia* grown on 2-tridecanone. *J. Bacteriol.*, 118:880–889.
97. Shum, A.C. and Markovetz, A.J., 1974, Specificity and induction of undecyl acetate esterase from *Pseudomonas cepacia* grown on 2-tridecanone. *J. Bacteriol.*, 118:890–897.
98. Sikkema, J., de Bont J.A.M., and Poolman, B., 1995, Mechanisms of membrane toxicity of hydrocarbons. *Microbiol. Rev.*, 59:201–222.
99. Sluis, M.K., Sayavedra Soto, L.A., and Arp, D.J., 2002, Molecular analysis of the soluble butane monooxygenase from '*Pseudomonas butanovora*'. *Microbiology*, 148:3617–3629.
100. Smits, T.H.M., Balada, S.B., Witholt, B., and van Beilen, J.B., 2002, Functional analysis of alkane hydroxylases from Gram-negative and Gram-positive bacteria. *J. Bacteriol.*, 184:1733–1742.
101. Smits, T.H.M., Röthlisberger, M., Witholt, B., and van Beilen, J.B., 1999, Molecular screening for alkane hydroxylase genes in Gram-negative and Gram-positive strains. *Environ. Microbiol.*, 1:307–318.
102. Smits, T.H.M., Seeger, M.A., Witholt, B., and van Beilen, J.B., 2001, New alkane-responsive expression vectors for *E. coli* and *Pseudomonas*. *Plasmid*, 46:16–24.
103. Smits, T.H.M., Witholt, B., and van Beilen, J.B., 2003, Functional characterization of genes involved in alkane oxidation by *Pseudomonas aeruginosa*. *Antonie van Leeuwenhoek*, 84:193–200.
104. Söhngen, N.L., 1913, Benzin, Petroleum, Paraffinöl und Paraffin als Kohlenstoff- und Energiequelle für Mikroben. *Zentr. Bacteriol. Parasitenk., Abt. II*, 37:595–609.
105. Sotsky, J.B., Greer, C.W., and Atlas, R.M., 1994, Frequency of genes in aromatic and aliphatic hydrocarbon biodegradation pathways within bacterial populations from Alaskan sediments. *Can. J. Microbiol.*, 40:981–985.
106. Staijen, I.E., Marcionelli, R., and Witholt, B., 1999, The *PalkBFGHJKL* promoter is under catabolite repression control in *Pseudomonas oleovorans* but not in *Escherichia coli alk⁺* recombinants. *J. Bacteriol.*, 181:1610–1616.
107. Staijen, I.E., van Beilen, J.B., and Witholt, B., 2000, Expression, stability and performance of the three-component alkane monooxygenase of *Pseudomonas oleovorans* in *Escherichia coli*. *Eur. J. Biochem.*, 267:1957–1965.
108. Stanier, R.Y., Palleroni, N.J., and Doudoroff, M., 1966, The aerobic pseudomonads: A taxonomic study. *J. Gen. Microbiol.*, 43:159–271.
109. Stapleton, R.D. and Sayler, G.S., 1998, Assessment of the microbiological potential for the natural attenuation of petroleum hydrocarbons in a shallow aquifer system. *Microb. Ecol.*, 36:349–361.
110. Stapleton, R.D., Sayler, G.S., Boggs, J.M., Libelo, E.L., Stauffer, T., and MacIntyre, W.G., 2000, Changes in subsurface catabolic gene frequencies during natural attenuation of petroleum hydrocarbons. *Environ. Sci. Technol.*, 34:1991–1999.
111. Sticher, P., Jaspers M.C.M., Stemmler, K., Harms, H., Zehnder, A.J.B., and van der Meer, J.R., 1997, Development and characterization of a whole-cell bioluminescent sensor for bioavailable middle-chain alkanes in contaminated groundwater samples. *Appl. Environ. Microbiol.*, 63:4053–4060.
112. Stover, C.K. et al., 2000, Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature*, 406:959–964.

113. Stutz, E.W., Défago, G., and Kern, H., 1986, Naturally occurring fluorescent *Pseudomonads* involved in suppression of black root rot of Tobacco. *Phytopathology*, 76:181–185.
114. Tani, A., Ishige, T., Sakai, Y., and Kato, N., 2001, Gene structures and regulation of the alkane hydroxylase complex in *Acinetobacter* sp. strain M-1. *J. Bacteriol.*, 183:1819–1823.
115. Thyse G.J.E. and van der Linden, A.C., 1958, N-alkane oxidation by a *Pseudomonas*. Studies on the intermediate metabolism. *Antonie van Leeuwenhoek*, 24:298–308.
116. Traxler, R.W. and Bernard, J.M., 1969, The utilization of *n*-alkanes by *Pseudomonas aeruginosa* under conditions of anaerobiosis. I. Preliminary observation. *Int. Biodeterior. Bull.*, 5:21–25.
117. van Beilen, J.B., Eggink, G., Enequist, H., Bos, R., and Witholt, B., 1992, DNA sequence determination and functional characterization of the OCT-plasmid encoded *alkJ/KL* genes of *Pseudomonas oleovorans*. *Mol. Microbiol.*, 6:3121–3136.
118. van Beilen, J.B., Kingma, J., and Witholt, B., 1994, Substrate specificity of the alkane hydroxylase of *Pseudomonas oleovorans* GPo1. *Enzyme Microb. Technol.*, 16:904–911.
119. van Beilen, J.B., Li, Z., Duetz, W.A., Smits T.H.M., and Witholt, B., 2003, Diversity of alkane hydroxylase systems in the environment. *Oil Gas Sci. Technol.*, 58:1–12.
120. van Beilen, J.B., Neuenschwander, M., Smits T.H.M., Roth, C., Balada, S.B., and Witholt, B., 2002, Rubredoxins involved in alkane oxidation. *J. Bacteriol.*, 184:1722–1732.
121. van Beilen, J.B., Panke, S., Lucchini, S., Franchini, A.G., Röthlisberger, M., and Witholt, B., 2001, Analysis of *Pseudomonas putida* alkane degradation gene clusters and flanking insertion sequences: Evolution and regulation of the *alk*-genes. *Microbiology*, 147:1621–1630.
122. van Beilen, J.B., Penninga, D., and Witholt, B., 1992, Topology of the membrane-bound alkane hydroxylase of *Pseudomonas oleovorans*. *J. Biol. Chem.*, 267:9194–9201.
123. van Beilen, J.B., Smits T.H.M., and Witholt, B., 2002, Directed evolution of the *Pseudomonas putida* GPo1 alkane hydroxylase system. *Abstr. Gen. M. Am. Soc. Microbiol.*, 102:418.
124. van Beilen, J.B., Veenhoff, L., and Witholt, B., 1998, Alkane hydroxylase systems in *Pseudomonas aeruginosa* strains able to grow on *n*-octane. In K. Kieslich, C.P. van der Beek, J.A.M. de Bont, W.J.J. van den Tweel (eds), *New Frontiers in Screening for Microbial Biocatalysts*, pp. 211–215. Elsevier Science, B.V., Amsterdam.
125. van Beilen, J.B., Wubbolts, M.G., Chen, Q., Nieboer, M., and Witholt, B., 1996, Effects of two-liquid-phase systems and expression of *alk* genes on the physiology of alkane-oxidizing strains. In T. Nakazawa, K. Furukawa, D. Haas, B. Witholt (eds) *Molecular Biology of Pseudomonads*. ASM Press, Washington DC.
126. van der Linden, A.C., 1963, Epoxidation of α -olefins by heptane-grown *Pseudomonas* cells. *Biochim. Biophys. Acta*, 77:157–159.
127. van der Linden, A.C. and Thijsse G.J.E., 1965, The mechanisms of microbial oxidations of petroleum hydrocarbons. *Adv. Enzymol.*, 27:469–545.
128. van der Linden, A.C. and van Ravenswaay Claasen, J.C., 1971, Hydrophobic enzymes in hydrocarbon degradation. *Lipids*, 6:437–443.
129. van Eyk, J. and Bartels, T.J., 1968, Paraffin oxidation in *Pseudomonas aeruginosa*. I. Induction of paraffin oxidation. *J. Bacteriol.*, 96:706–712.
130. van Eyk, J. and Bartels, T.J., 1970, Paraffin oxidation in *Pseudomonas aeruginosa*. II. Gross fractionation of the enzyme system into soluble and particulate components. *J. Bacteriol.*, 104:1065–1073.
131. van Ravenswaay Claasen, J.C., and van der Linden, A.C., 1971, Substrate specificity of the paraffin hydroxylase of *Pseudomonas aeruginosa*. *Antonie van Leeuwenhoek*, 37:339–352.
132. Vandecasteele, J.P., Blanchet, D., Tassin, J.P., Bonamy, A.M., and Guerrillot, L., 1983, Enzymology of alkane degradation in *Pseudomonas aeruginosa*. *Acta Biotechnol.*, 3:339–344.

133. Vomberg, A. and Klinner, U., 2000, Distribution of *alkB* genes within n-alkane-degrading bacteria. *J. Appl. Microbiol.*, 89:339–348.
134. Whyte, L.G., Greer, C.W., and Inniss, W.E., 1996, Assessment of the biodegradation potential of psychrotrophic microorganisms. *Can. J. Microbiol.*, 42:99–106.
135. Whyte, L.G., Smits T.H.M., Labbé, D., Witholt, B., Greer, C.W., and van Beilen, J.B., 2002, Cloning and characterization of multiple alkane hydroxylase systems in *Rhodococcus* spp. strains Q15 and 16531. *Appl. Environ. Microbiol.*, 68:5933–5942.
136. Wubbolts, M.G., 1994, Xylene and alkane mono-oxygenases from *Pseudomonas putida*. Ph.D. thesis. University of Groningen, Groningen, The Netherlands.
137. Yuste, L., Canosa, I., and Rojo, F., 1998, Carbon-source-dependent expression of the *PalkB* promoter from the *Pseudomonas oleovorans* alkane degradation pathway. *J. Bacteriol.*, 180:5218–5226.
138. Yuste, L. and Rojo, F., 2001, Role of the *crc* gene in catabolic repression of the *Pseudomonas putida* GPo1 alkane degradation pathway. *J. Bacteriol.*, 183:6197–6206.
139. Zobell, C.E., 1950, Assimilation of hydrocarbons by microorganisms. *Adv. Enzymol.*, 10:443–486.

GENOMIC INSIGHTS IN THE METABOLISM OF AROMATIC COMPOUNDS IN *PSEUDOMONAS*

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1. INTRODUCTION

Pseudomonads are ubiquitous γ -proteobacteria with a remarkable degree of physiological and genetic adaptability. Members of the genus *Pseudomonas* are found in large numbers in different natural environments (soil, freshwater, marine) as well as in association with plants and animals. These bacteria are involved in important metabolic activities in the environment, being element cycling and degradation of biogenic and xenobiotic pollutants some of their major tasks^{56, 64, 90, 96}. The metabolic versatility of *Pseudomonas* strains has been used for biotechnological applications, mainly to degrade waste (bioremediation) and to synthesize specialty chemicals (biocatalysis)^{69, 99}.

Next to glucosyl residues, the benzene ring is the most widely distributed unit of chemical structure in nature. Moreover, the thermodynamic stability of the benzene ring increases its persistence in the environment¹⁴. Aromatic compounds are also major environmental pollutants and a significant number of xenobiotics (e.g., polychlorinated biphenyls, polychlorinated

dioxins, nitroaromatics, etc.) belong to this family of compounds^{33, 46, 77}. The catabolism of aromatic compounds involves a wide variety of peripheral pathways that channel structurally diverse substrates into a limited number of common intermediates which are further processed by a few central pathways to Krebs cycle intermediates^{33, 46, 77}. In the aerobic catabolic funnel most of the peripheral pathways converge on dihydroxy aromatic compounds (catechol, protocatechuate, gentisate, homoprotocatechuate, homogentisate, hydroquinone, hydroxyquinol) which are the substrate of ring-cleavage enzymes that open the aromatic ring³³. Aerobic degradation of phenylacetic acid is an exception since it requires formation of phenylacetyl-CoA as central intermediate⁴⁸. Figure 1 illustrates the catabolic potential of *Pseudomonas* to funnel a broad range of aromatic compounds, some of them important environmental pollutants, into the central metabolism. Whereas peripheral pathways involved in degradation of xenobiotic compounds, such as those carrying halogen atoms or nitro groups, are only present in certain *Pseudomonas* strains^{77, 87}, those involved in the catabolism of common aromatic compounds, such as benzoate or aromatic amino acids, are widespread. The general ability of *Pseudomonas* strains to use aromatic compounds as sole carbon source is related to the fact that most of these compounds are commonly present in the environment as a result of the recycling of plant-derived material³⁶.

In this chapter we perform a chromosomal search and provide a global view on the catabolism of aromatic compounds by the *Pseudomonas* strains whose genomes are partially (*Pseudomonas fluorescens* and *Pseudomonas syringae*) or totally (*Pseudomonas putida* and *Pseudomonas aeruginosa*) known.

P. putida, a non-pathogenic member of rRNA group I of the genus *Pseudomonas*, is the best characterized saprophytic Pseudomonad and the genome of strain KT2440 (6, 181, 803 bp) has been recently sequenced⁵⁶. This bacterium is a TOL plasmid-free⁴, spontaneous restriction-deficient derivative of *P. putida* mt-2^{3, 25}, a strain that was isolated from soil by its ability to use *m*-toluate as the sole carbon source, a feature later shown to be due to the presence of the TOL plasmid pWW0^{2, 59}. *P. putida* KT2440 is mainly known by its ability to degrade aromatic compounds and for being an ideal host for expanding the range of substrates that it can degrade and/or biotransform in added-value products through the recruitment of genes from other microorganisms^{32, 74, 94}. *P. putida* KT2440 is able to grow in minimal medium containing benzoate, 4-hydroxybenzoate, benzylamine, phenylacetate, phenylalanine, tyrosine, phenylethylamine, phenylhexanoate, phenylheptanoate, phenyloctanoate, coniferyl alcohol, 4-coumarate, 4-hydroxyphenylpropionate, ferulate, caffeate, vanillate, nicotinate and quinate (hydroaromatic compound) as sole carbon and energy sources⁴⁵.

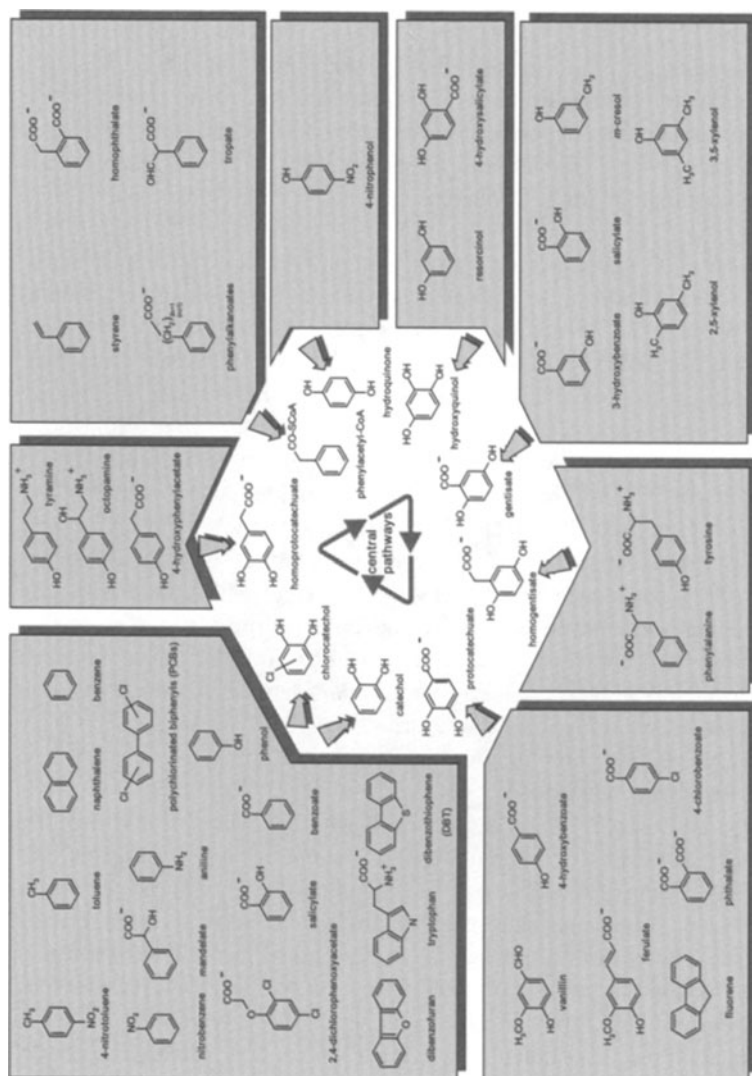


Figure 1. General view of the aerobic catabolism of natural and xenobiotic aromatic compounds in *Pseudomonas*. A broad range of aromatic compounds (boxed) funnel through a wide variety of peripheral pathways (represented by thick arrows) into a limited number of common intermediates (catechol, chlorocatechol, protocatechuate, homogentisate, gentisate, hydroxyquinol, hydroquinone, homoprotocatechuate, phenylacetyl-CoA), which are then processed by the cognate central pathways to Krebs cycle intermediates.

P. aeruginosa PAO1 is a major opportunistic human pathogen due to its resistance to antibiotics and disinfectants. This bacterium is the predominant cause of morbidity and mortality in cystic fibrosis patients. In addition, *P. aeruginosa* is a normal inhabitant of the environment and it grows in soil, marshes and coastal marine habitat, as well as on plant tissues. The genome of *P. aeruginosa* PAO1 (6,264,403 bp) was the first one to be reported within the genus *Pseudomonas*⁹¹. *P. aeruginosa* PAO1 (strain 131 of Stanier *et al.*, 1966) uses L-mandelate, benzoylformate, benzoate, 4-hydroxybenzoate, quinate, tyrosine, phenylalanine, tryptophan, L-kynurenine, anthranilate, tyramine, dopamine, octopamine, synephrine, norepinephrine, 4-hydroxyphenylacetic acid, vanillylmandelic acid, homogentisate and gentisate as sole carbon sources^{13, 89}.

P. fluorescens encompasses a group of common, non-pathogenic saprophytes that colonize soil, water and plant surface environments. In addition to its ability to degrade a wide variety of organic compounds, *P. fluorescens* strains produce a number of secondary metabolites and they have been used as biocontrol agents of plant pathogens and as plant-growth promoting bacteria¹⁰⁰. The genome of three strains of *P. fluorescens*, strain Pf0-1, Pf-5 and SBW25, are being sequenced by the Joint Genome Institute (http://www.jgi.doe.gov/JGI_microbial/html/pseudomonas/pseudo_facts.html), The Institute for Genomic Research (http://tigrblast.tigr.org/ufmg/index.cgi?database=p_fluorescens|seq) and the Sanger Institute (http://www.sanger.ac.uk/Projects/P_fluorescens/), respectively. *P. syringae* is a plant pathogen that has negative effects on both food and biomass production. Moreover, many strains of this species have been exploited for industrial purposes such as active ice nuclei catalyzers³⁹. The genomes of *P. syringae* pv. *tomato* DC3000 (<http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/framik?db=Genome&gi=279>) and *P. syringae* B728a (http://genome.jgi-psf.org/draft_microbes/psesy/psesy.home.html) are being sequenced.

While the first part of this chapter deals with the gene clusters encoding the central pathways for degradation of aromatic compounds in the *Pseudomonas* strains reported above, the second part deals with the gene clusters responsible for the peripheral pathways that funnel into the central routes. Some general conclusions are presented at the end of the chapter. Since the aim of this work is to provide a general view on the aerobic catabolism of aromatics derived from the *in silico* analyses of the genomes of some *Pseudomonas* strains, a detailed characterization of each catabolic pathway will not be addressed. The biochemical and genetic features of the aerobic pathways for the catabolism of aromatics have been revised in a series of review articles^{33, 36, 77}, in the Biocatalysis/Biodegradation Database (<http://umbbd.ahc.umn.edu/>) as well as in other chapters of this volume.

2. CENTRAL PATHWAYS FOR THE CATABOLISM OF AROMATICS

A genomic search in the chromosome of the *Pseudomonas* strains that were subject of study (see above) revealed the existence of at least five major central pathways for the catabolism of aromatic compounds. The location and arrangement of the gene clusters encoding such central pathways in the complete chromosome of *P. putida* KT2440 and *P. aeruginosa* PAO1 is indicated in Figure 2.

2.1. The β -Ketoadipate Pathway

Considering the ubiquity of the β -ketoadipate pathway (*ortho*-cleavage pathway) and its key role in the catabolism of a wide variety of aromatic compounds (Figure 1), it is not surprising that this pathway is present in all four *Pseudomonas* species whose genomes have been analyzed. The two branches of the β -ketoadipate pathway, that is, the protocatechuate branch (*pca* genes) and the catechol branch (*cat* genes), converge at β -ketoadipate enol-lactone in *Pseudomonas*, and one set of enzymes (*pcaDIJF* gene products) complete the conversion of the latter to the Krebs cycle intermediates, succinate and acetyl-CoA (Figure 3)³⁶.

2.1.1. The Protocatechuate Branch

Protocatechuate is a key intermediate in the catabolism of a number of natural and xenobiotic aromatic compounds (Figure 1). The *pca* genes encode the protocatechuate branch of the β -ketoadipate pathway³⁶ (Figure 3).

While the *pca* genes are organized in two different clusters in *P. aeruginosa*, their orthologs in *P. putida* and *P. syringae* are arranged in three different clusters (Figure 4). The spread of *pca* genes in different clusters within the bacterial chromosome has been also observed in some β -proteobacteria such as *Burkholderia pseudomallei* and *Ralstonia metallidurans*⁴⁵. In contrast, the catabolic *pca* genes from *P. fluorescens* are arranged in a single cluster (Figure 4), which resembles the single *pca* gene cluster found in *Acinetobacter* sp. ADP1³⁶, in the α -proteobacteria *Agrobacterium tumefaciens* and *Caulobacter crescentus*, as well as in the gram-positive nocardioform actinomycete *Rhodococcus opacus*^{21, 45}.

The two pairs of genes *pcaHG* and *pcaIJ* encode the separate subunits of the protocatechuate 3,4-dioxygenase and the β -ketoadipate succinyl-CoA transferase, respectively (Figure 3), and they are co-transcribed in different bacteria³⁶. The *pcaHG* gene products in *P. fluorescens* and the *pcaIJ* gene products in *P. putida* show the lowest amino acid sequence similarity among

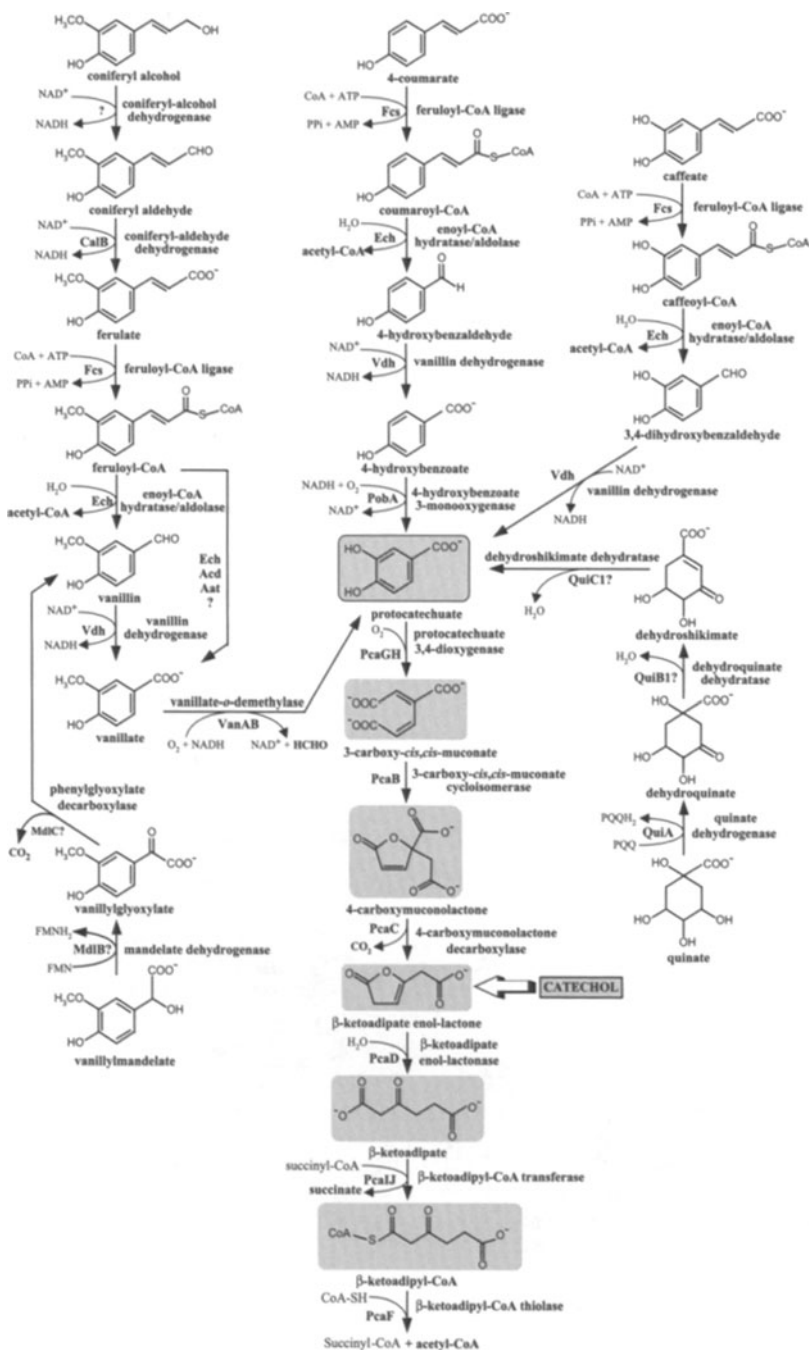


Figure 3. Peripheral pathways funneling to the protocatechuate branch of the β -ketoadipate pathway. The enzymes and metabolites involved in each biochemical step are indicated. ?, means an unknown or putative enzyme. The thick arrow indicates the convergence of the catechol and protocatechuate branches of the β -ketoadipate pathway. The protocatechuate central route is shaded.

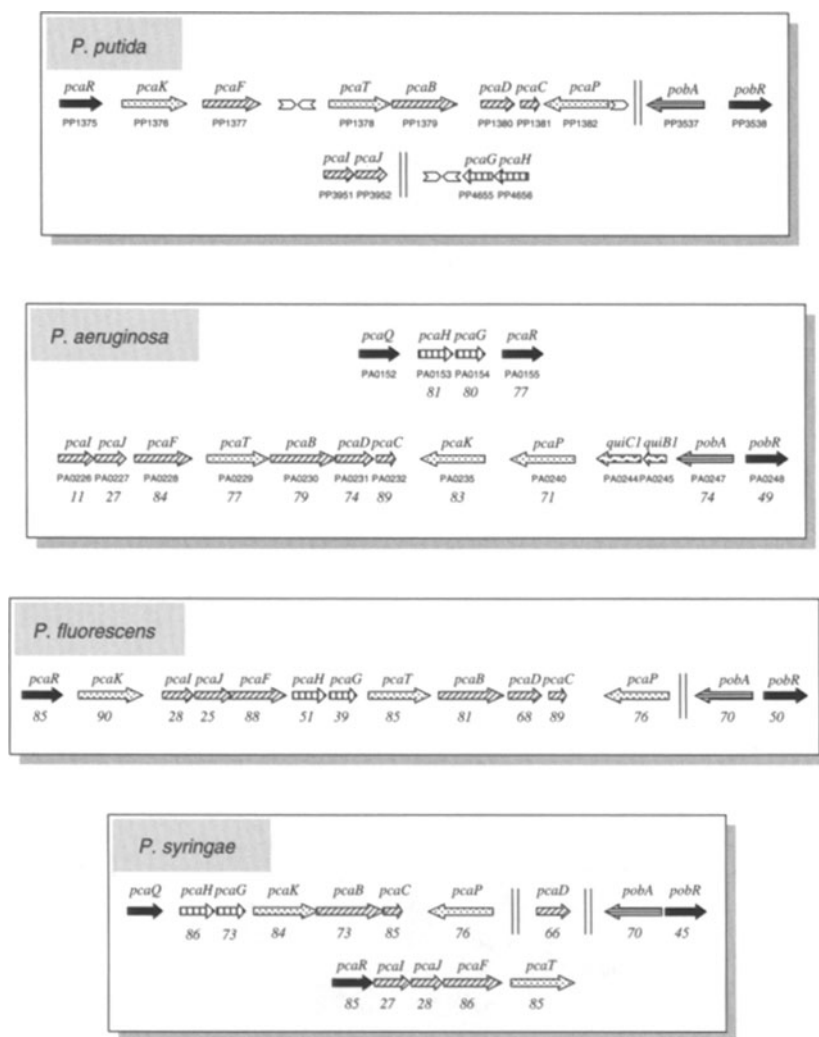


Figure 4. Gene arrangement of the *pob* and *pca* clusters in *Pseudomonas*. Genes are represented by arrows: black (regulatory genes), stippled (transport genes), vertically striped (genes encoding the protocatechuate 3,4-dioxygenase), horizontally striped (genes encoding the 4-hydroxybenzoate monooxygenase), hatched (catabolic genes of the β -ketoadipate pathway), cross-hatched (*quiBIC1* genes). Arrowheads represent the *P. putida* repetitive extragenic palindromic (REP) element. Vertical lines indicate that the genes are not adjacent in the genome. PP and PA numbers below the genes indicate the corresponding proteins in the annotated genomes of *P. putida* and *P. aeruginosa*, respectively. Numbers underneath the arrows indicate the percentage of amino acid sequence identity between the encoded gene product and the equivalent product from *P. putida* KT2440 (adapted from ref. [45]).

pca gene products of different *Pseudomonas* strains, and this may reflect a different evolutionary origin for these pairs of genes in these bacteria. Thus, the *pcaIJ* genes from *P. putida* might have been originated in a non *Pseudomonas* strain since they are organized in a single cluster and they are more similar to orthologs from other proteobacteria, for example, *Acinetobacter*, *Ralstonia* and *Burkholderia*⁴⁵, than to the equivalent *pcaIJ* genes from other *Pseudomonas* species (Figure 4). Although in *P. aeruginosa* and *P. syringae* there are additional *pcaIJ*-like gene products (not shown in Figures 2 and 4) that show about 50% amino acid sequence identity with *pcaIJ* gene products from *P. putida*, the corresponding genes are not linked to the *pca* gene cluster⁴⁵ and, therefore, it is uncertain whether they are involved in the protocatechuate branch or they encode other CoA transferase activities such as those reported in degradation of dicarboxylic acids^{40, 68}.

The *pcaR* gene encodes a transcriptional regulator (IcIR-type regulatory protein) of the *pca* genes and its location within the *pca* cluster differs among the four *Pseudomonas* species (Figure 4). In *P. putida*, the PcaR activator was shown to control the β -ketoadipate-dependent inducible expression of genes *pcaRKFTBDCP* and *pcaIJ*, which are required for the conversion of β -carboxy-*cis,cis*-muconate to Krebs cycle intermediates^{28, 36}. A gene (*pcaQ*) encoding a putative LysR-type regulator homologous to the PcaQ activator from *A. tumefaciens*⁶⁶ is found in the vicinity of the *pcaHG* genes in *P. aeruginosa* and *P. syringae* (Figure 4) and, therefore, it could behave as the regulator controlling the expression of *pcaHG* in these *Pseudomonas* strains.

The *pcaK* and *pcaT* genes encode proteins of the major facilitator superfamily (MFS) for transport of 4-hydroxybenzoate and β -ketoadipate, respectively, in *P. putida* PRS2000^{36, 67}. These two genes are present in the *pca* clusters of the four *Pseudomonas* species (Figure 4). A putative porin-encoding gene (*pcaP*) is also found within the *pca* clusters (Figure 4). In *P. putida* KT2440, *pcaP* is adjacent to the *tigCBA* genes (PP1384, PP1385 and PP1386, respectively) encoding a solvent efflux pump²⁶, an arrangement similar to that observed in the solvent tolerant *P. putida* DOT-T1 strain⁷⁵.

2.1.2. The Catechol Branch

Catechol is a common intermediate during degradation of a significant number of aromatic compounds (Figure 1). The *cat* genes encode proteins responsible of catechol degradation and they are usually organized in a single cluster³⁶. In *Pseudomonas*, the *cat* genes encode the catechol 1,2-dioxygenase (CatA) that cleaves catechol in *ortho* position, and the two enzymes (CatBC) that transform the ring-cleavage product, *cis,cis*-muconate, into β -ketoadipate enol-lactone^{36, 47} (Figure 5). Conversion of the latter to Krebs cycle intermediates is carried out by the products of the *pcaD*, *pcaIJ* and *pcaF* genes

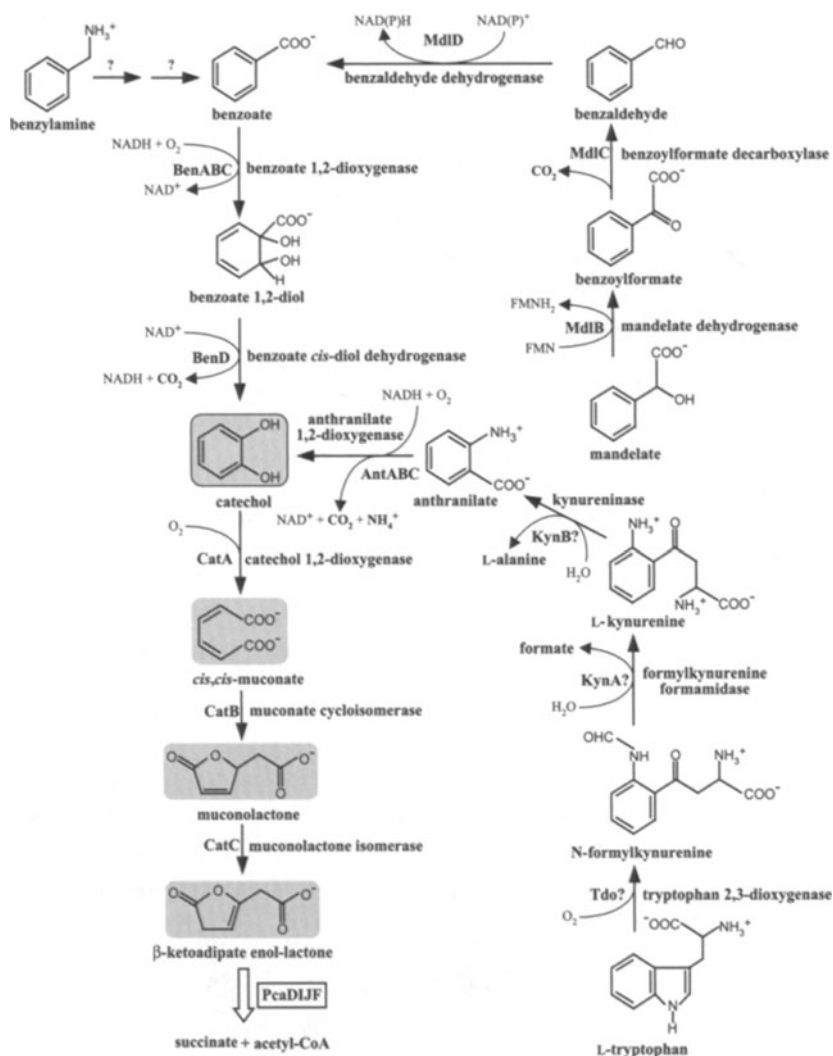


Figure 5. Peripheral pathways funneling to the catechol branch of the β -ketoadipate pathway. The enzymes and metabolites involved in each biochemical step are indicated. ?, means an unknown or putative enzyme. The thick arrow indicates that the last biochemical steps of the catechol branch are carried out by the PcaDIJF enzymes of the protocatechuate branch (see Figure 3). The catechol central route is shaded.

(Figures 3 and 5). In some bacterial species, such as *Acinetobacter* sp. ADP1, the catechol branch and the protocatechuate branch never converge and two independently regulated set of genes encode isofunctional enzymes for the last three steps of the pathway³⁶.

Whereas the *catRBCA* gene order is maintained in the single *cat* cluster of *P. putida*³⁶ and *P. aeruginosa* (this arrangement differs from the *catCBA* order given by Kukor *et al.* [1988] and Zhang *et al.* [1993]), *P. fluorescens* has two sets of *catBCA* genes that lack the *catR* regulatory gene (Figure 6). So far, no *cat* cluster has been found in *P. syringae*. Interestingly, a second *catA* gene (named *catA2*) that is present within the *ben* cluster for benzoate degradation (see Section 3.2.1) was found in *P. putida* KT2440 (Figure 6)⁴⁵. The *catA2* gene is not present in the *ben* clusters from other *Pseudomonas* strains such as *P. putida* PRS2000¹¹, *P. aeruginosa* and *P. fluorescens* (Figure 6). However, it is known that *P. arvilla* C-1 (later reclassified as *P. putida*) has three functional isoenzymes ($\alpha\alpha$, $\alpha\beta$ and $\beta\beta$) of catechol 1,2-dioxygenase, being the α and

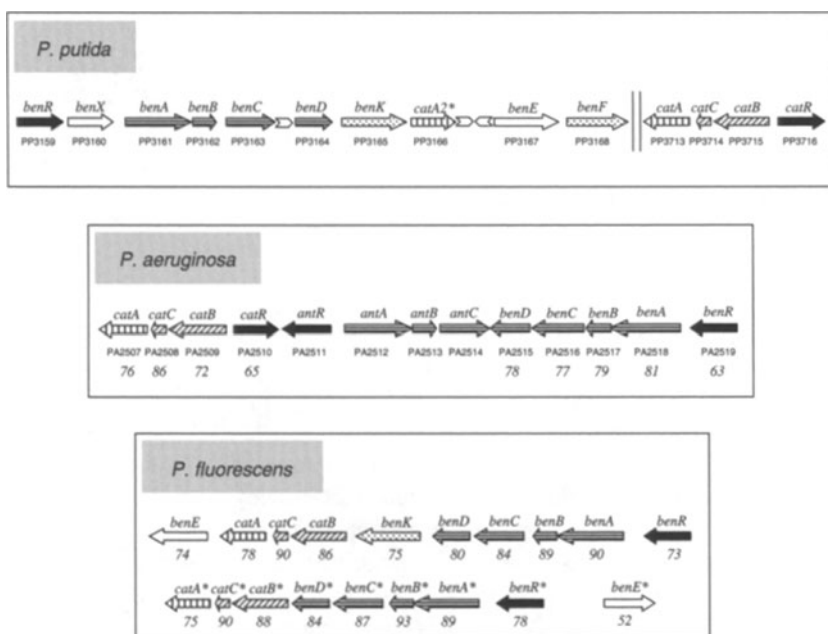


Figure 6. Gene arrangement of the *ben*, *cat* and *ant* clusters in *Pseudomonas*. Genes are represented by arrows: black (regulatory genes), stippled (transport genes), vertically striped (genes encoding the catechol 1,2-dioxygenase), horizontally striped (genes encoding the benzoate dioxygenase/dihydrodiol dehydrogenase and the anthranilate dioxygenase), hatched (catabolic genes of the catechol branch), white (genes of unknown function). Asterisks indicate a second copy of the gene in the genome. Arrowheads represent the *P. putida* REP element. Vertical lines indicate that the genes are not adjacent in the genome. PP and PA numbers below the genes indicate the corresponding proteins in the annotated genomes of *P. putida* and *P. aeruginosa*, respectively. Numbers underneath the arrows indicate the percentage of amino acid sequence identity between the encoded gene product and the equivalent product from *P. putida* KT2440 (adapted from ref. [45]).

β subunits encoded by the *catA $_{\alpha}$* and *catA $_{\beta}$* genes, respectively⁵⁵. While the *catA $_{\beta}$* gene is homologous to the *catA* gene from *P. putida* KT2440, the N-terminal sequence of the α subunit of catechol 1,2-dioxygenase from *P. arvilla* C-1⁵⁵ is homologous to the deduced N-terminal sequence of CatA2 from *P. putida* KT2440, suggesting that *catA2* might encode an active catechol 1,2-dioxygenase not reported yet in this strain. Certain bacteria such as *Frateriia* sp. strain ANA-18, *Acinetobacter lwoffii* K24, and *Burkholderia* sp. strain TH2 also possess two sets of catechol 1,2-dioxygenases, CatA1 and CatA2⁹². Although the significance of possessing two sets of CatA enzymes is still unclear, the second copy of the *catA* gene could have several functions such as avoiding accumulation of toxic catechol, facilitating the rapid formation of the inducer (muconate) of the *cat* cluster, and increasing the range of catechol derivatives that can be *ortho* cleaved.

The expression of the *catBCA* cluster in *P. putida* is controlled by the CatR activator (LysR-type regulatory protein) in response to the inducer *cis,cis*-muconate⁸⁰. A *catR* homolog that is transcribed divergently with respect to the cognate *catBCA* catabolic genes is also found in *P. aeruginosa* but is lacking in the two *cat* clusters from *P. fluorescens*. The gene arrangement in *P. fluorescens*, as well as that of the *catA2* gene in *P. putida* KT2440, suggests that such *cat* genes could be under the control of the BenR regulator (see Section 3.2.1; Figure 6).

2.2. The Phenylacetyl-CoA Pathway

The *pha* genes encode the proteins involved in phenylacetate degradation in *P. putida* (Figure 7)⁴⁸. The catabolism of phenylacetic acid represents a novel hybrid pathway which does not follow the conventional routes for aerobic biodegradation of aromatic compounds, that is, activation of the aromatic ring by formation of dihydric phenols (aromatic compounds carrying two hydroxyl groups) such as catechol or protocatechuate. On the contrary, the aerobic catabolism of phenylacetic acid in *Pseudomonas* and other bacteria is initiated by a phenylacetyl-CoA ligase (PhaE) that activates phenylacetic acid to phenylacetyl-CoA, an enzymatic reaction that resembles the CoA thioesterification of the aromatic ring in the anaerobic catabolism of aromatic compounds³⁴. Then, phenylacetyl-CoA suffers an oxygenation reaction, cleavage of the aromatic ring and a β -oxidation-like pathway of the ring cleavage product^{23, 48, 53} (Figure 7). So far, details on the enzymatic mechanisms and nature of the intermediates generated during the catabolism of phenylacetyl-CoA are still unknown.

The *pha* cluster from *P. putida* is organized in four discrete DNA segments (Figure 8A) which are predicted to encode six different functional

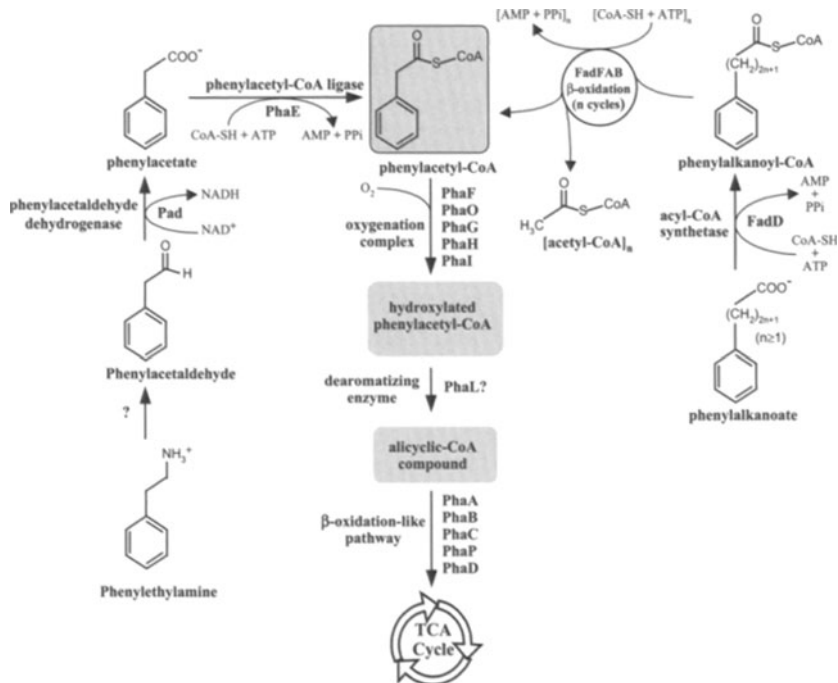
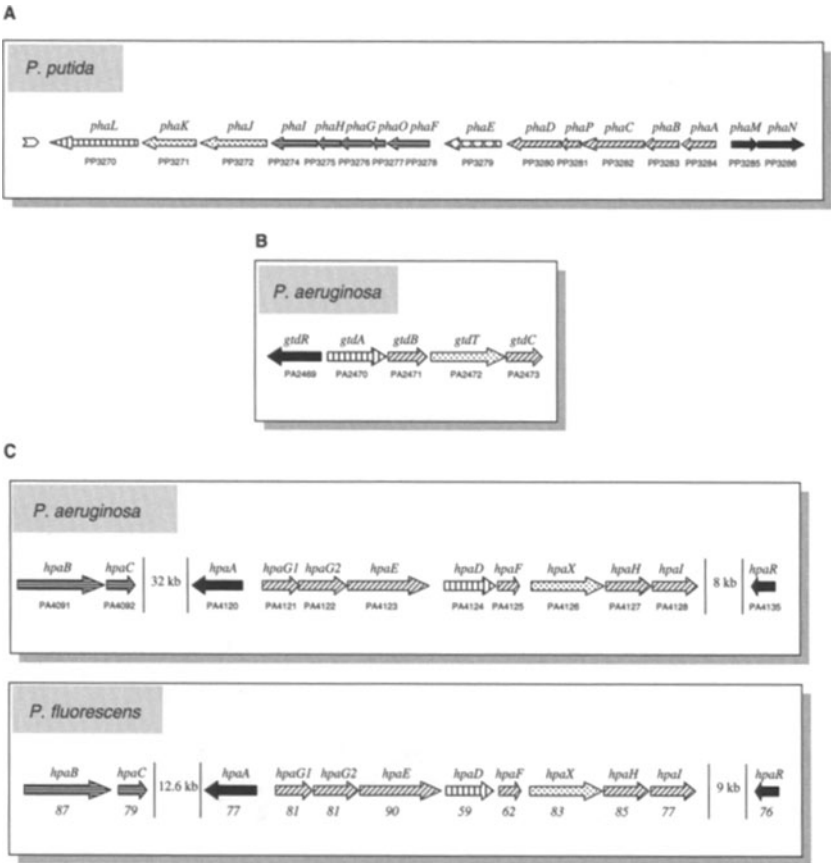


Figure 7. Peripheral pathways funneling to the phenylacetyl-CoA pathway. The enzymes and metabolites involved in each biochemical step are indicated. ?, means an unknown or putative enzyme. TCA, indicates the Krebs cycle. The phenylacetyl-CoA central pathway is shaded.

units: *phaABCPD* and *phaE* (β -oxidation-like pathway and CoA activation of phenylacetic acid, respectively), *phaFOGHI* (hydroxylation of the aromatic ring), *phaJK* and *phaL* (phenylacetic acid transport and putative dearomatization of the ring, respectively) and *phaMN* (regulation of the *pha* cluster). The gene arrangement of the *pha* cluster in *P. putida* is different from that found in other bacteria, suggesting that various DNA rearrangements have occurred during the evolution of such cluster in each particular host^{45, 48, 53}. The β -oxidation-like functional unit encoded by the *phaABCPD* genes shows the highest diversity and it is absent in some bacteria, suggesting that the missing gene products may be replaced by similar enzymes from other β -oxidation pathways in the cell. The *phaJ* and *phaK* genes from *P. putida*, which encode a permease and a porin for the uptake of phenylacetic acid, respectively, are absent in the *pha* clusters of most bacteria⁴⁵. It is worth noting that no *pha* cluster is present in the *P. aeruginosa*, *P. fluorescens* and *P. syringae* strains analyzed, which agrees with previous studies revealing that most *P. aeruginosa* and *P. fluorescens* strains do not use phenylacetate as carbon source⁸⁹.



The regulatory *phaN* gene encodes a transcriptional repressor of the GntR family that controls the expression of the *pha* catabolic genes, being phenylacetyl-CoA the true inducer of the pathway²⁷. Although *phaM* is linked to the regulatory *phaN* gene in most bacteria^{45, 48, 53}, the physiological role of the *phaM* gene is still unknown.

2.3. The Homogentisate Pathway

Homogentisate is the central metabolite formed during degradation of some aromatic amino acids (Figure 1). The *hmgABC* genes from *Pseudomonas* are homologous to genes encoding the homogentisate dioxygenase (HmgA), maleylacetoacetate isomerase (Mai or HmgC) and fumarylacetoacetate hydrolase (Fah or HmgB) that convert homogentisate into fumarate and acetoacetate (Figure 9) in *Sinorhizobium meliloti*⁵¹ and *Emericella nidulans*²².

A regulatory gene, *hmgR*, is divergently transcribed from the *hmgABC* catabolic genes (Figure 10) and it encodes a repressor of the IclR family of transcriptional regulators. Whereas the gene arrangement within the *hmg* clusters from *P. putida*, *P. aeruginosa* and *P. fluorescens* is similar, the gene order is different in the *hmg* cluster from *P. syringae* (Figure 10). A gene (*hmgT*) encoding a potential transport protein of the MFS is located downstream of the *hmgC* gene in the genomes of *P. aeruginosa* and *P. fluorescens* (Figure 10).

2.4. The Gentisate Pathway

Gentisate (2,5-dihydroxybenzoate) is a key intermediate in the aerobic pathways for the catabolism of a number of aromatic compounds such as 3-hydroxybenzoate, 2-hydroxybenzoate (salicylate) and some phenol derivatives (Figure 1). Ring cleavage of gentisate is catalyzed by a gentisate 1,2-dioxygenase (GtdA) to form maleylpyruvate, which is then isomerized to fumarylpyruvate via a glutathione-dependent maleylpyruvate isomerase (GtdC). Finally, a fumarylpyruvate hydrolase (GtdB) hydrolyzes fumarylpyruvate to fumarate and pyruvate (Figure 11)¹⁰⁵.

The *gtdABC* genes from *P. aeruginosa* show significant similarity to the equivalent *nag* genes from *Ralstonia* sp. strain U2 that encode the gentisate degradation pathway in this bacterium¹⁰⁵. Therefore, the *gtdRABTC* cluster from *P. aeruginosa* (Figure 8B) may be involved in gentisate catabolism. No similar *gtd* genes were found in the genome of the other three *Pseudomonas* species. The *gtdR* gene is divergently transcribed with respect to the catabolic genes (Figure 8B) and it is likely to encode a LysR-type transcriptional regulator. A putative transport gene (*gtdT*) is located between the *gtdB* and *gtdC* catabolic genes (Figure 8B).

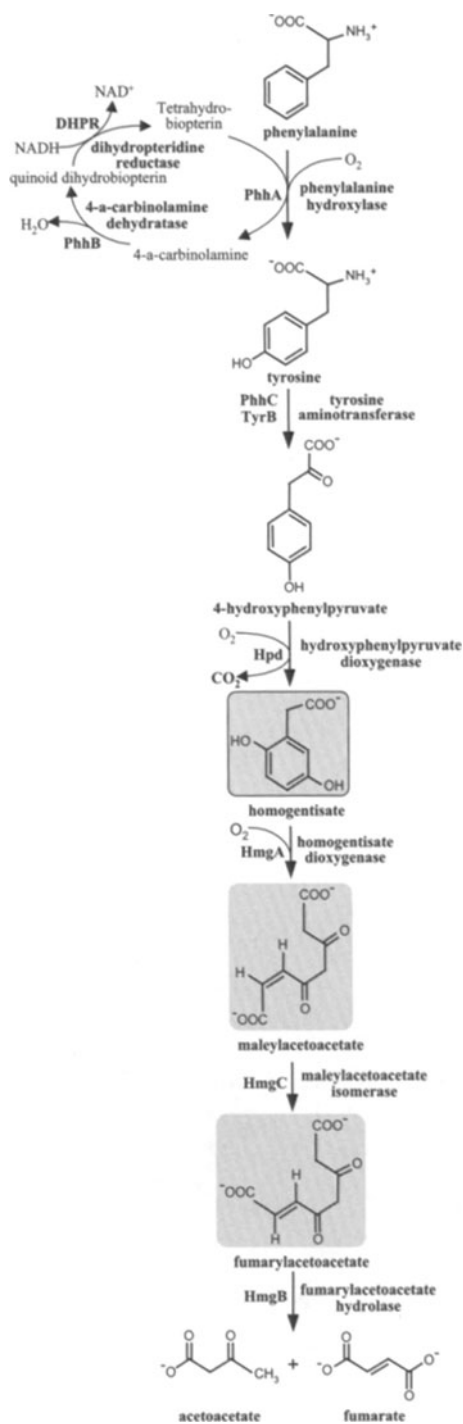


Figure 9. Peripheral pathways funneling to the homogentisate pathway. The enzymes and metabolites involved in each biochemical step are indicated. The homogentisate central pathway is shaded.

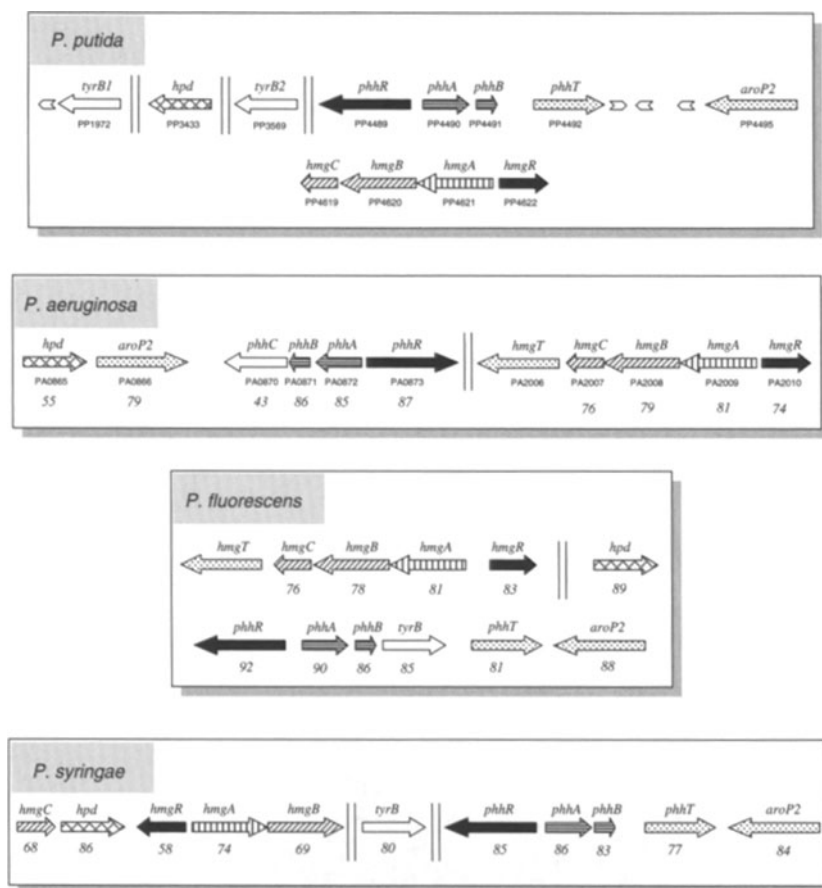


Figure 10. Gene arrangement of the *hmg* and *phh* clusters in *Pseudomonas*. Genes are represented by arrows: black (regulatory genes), stippled (transport genes), vertically striped (genes encoding the homogentisate dioxygenase), horizontally striped (genes encoding the phenylalanine hydroxylase), hatched (catabolic genes of the homogentisate pathway), white (genes encoding aromatic amino acid aminotransferases), cross-hatched (genes encoding the 4-hydroxyphenylpyruvate dioxygenase). Arrowheads represent the *P. putida* REP element. Vertical lines indicate that the genes are not adjacent in the genome. PP and PA numbers below the genes indicate the corresponding proteins in the annotated genomes of *P. putida* and *P. aeruginosa*, respectively. Numbers underneath the arrows indicate the percentage of amino acid sequence identity between the encoded gene product and the equivalent product from *P. putida* KT2440. Values underneath *tyrB* and *phhC* genes were obtained by comparison with the *tyrB1* gene product of *P. putida* KT2440. (adapted from ref. [45]).

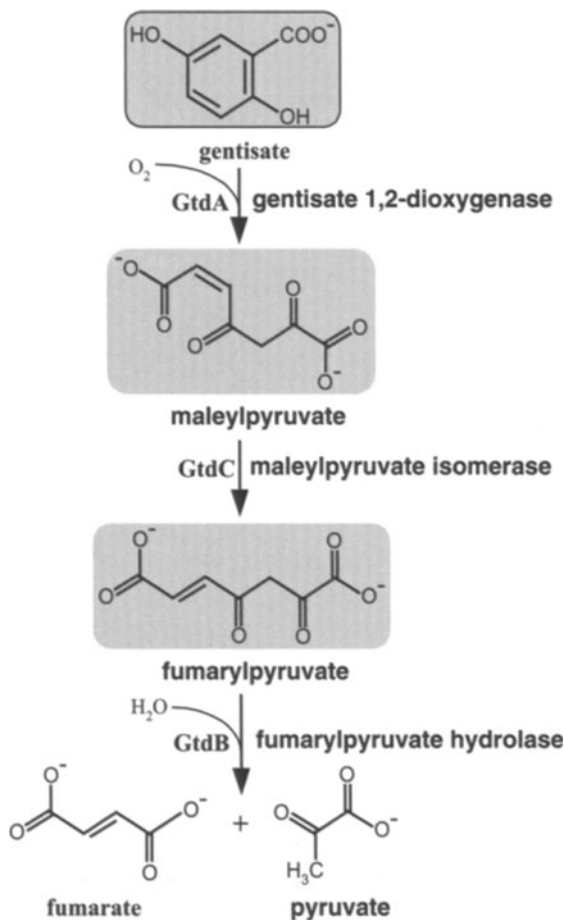
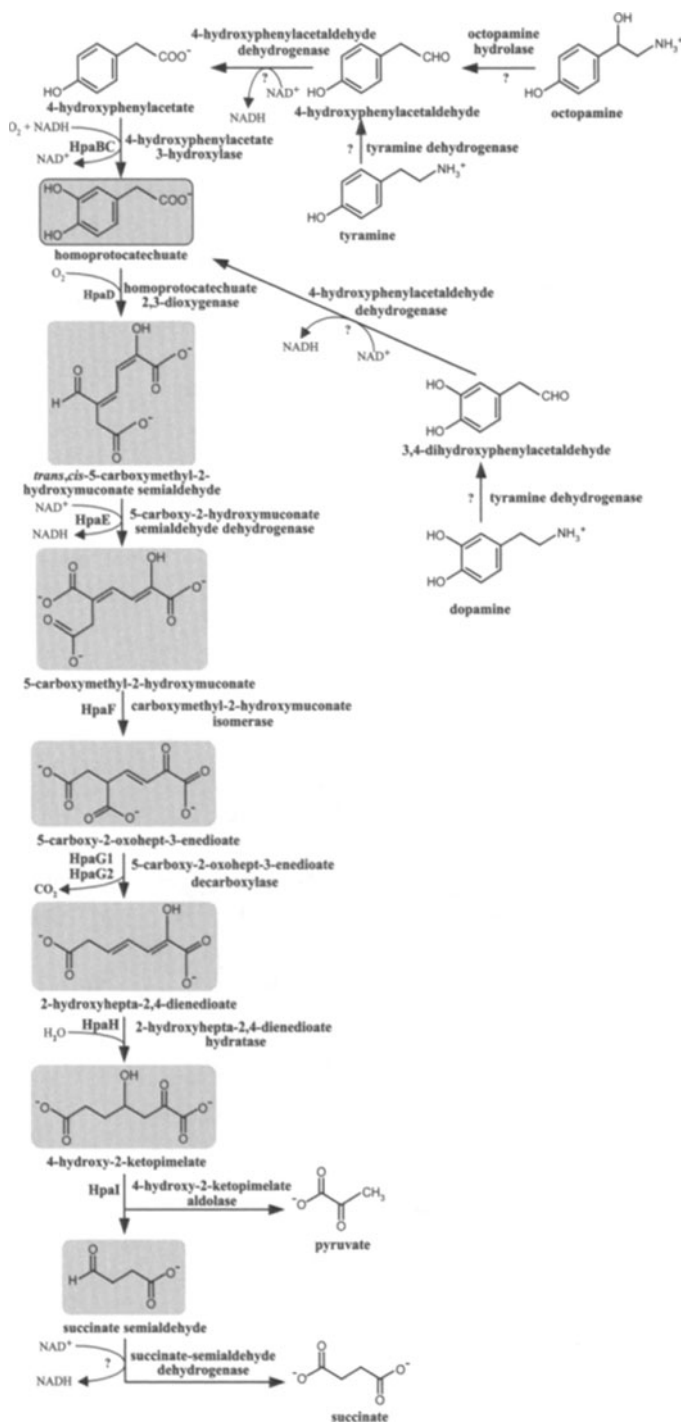


Figure 11. The gentisate pathway. The enzymes and metabolites involved in each biochemical step are indicated.

2.5. The Homoprotocatechuate Pathway

Homoprotocatechuate (3,4-dihydroxyphenylacetate) is the central intermediate in the catabolism of some hydroxylated aromatic acids and amines (Figure 1). In most bacteria, homoprotocatechuate becomes degraded via *meta*-cleavage of the aromatic ring by a homoprotocatechuate 2,3-dioxygenase. The resulting product (5-carboxymethyl-2-hydroxymuconic semialdehyde) is then converted into Krebs cycle intermediates via a dehydrogenative route (Figure 12)¹⁶.



P. aeruginosa is able to degrade homoprotocatechuate via a *meta*-cleavage pathway¹², and contains a gene cluster (Figure 8C) that shows significant similarity to the *hpa* cluster involved in homoprotocatechuate degradation in different microorganisms such as in enteric bacteria¹⁶. A similar *hpa* cluster is also found in *P. fluorescens* (Figure 8C). However, the gene arrangement within the *hpa* clusters from *Pseudomonas* strains is different from that observed in Enterobacteriaceae¹⁶. Thus, whereas the *hpaR* regulatory gene (encodes a transcriptional repressor of the MarR family) is located within the *hpa* cluster in enteric bacteria, the *hpaR* ortholog in *P. aeruginosa* and *P. fluorescens* is located outside of the cognate *hpa* cluster (Figure 8C). On the other hand, whereas the *hpaX* gene encoding a 4-hydroxyphenylacetic acid transport protein is located outside the *hpa meta*-cleavage operon in enteric bacteria¹⁶, the equivalent *hpaX* gene is located within such operon in *Pseudomonas* strains (Figure 8C). Interestingly, the *hpaG1* and *hpaG2* gene products from *Pseudomonas* are highly similar and they could be involved in the same enzymatic step (Figure 12) that is carried out by the HpaG protein in *E. coli*. Since the primary structure of HpaG reveals two similar domains, it is tempting to speculate that *hpaG1* and *hpaG2* genes from *Pseudomonas* have evolved as the two halves of *hpaG* in enteric bacteria¹⁶.

3. PERIPHERAL PATHWAYS FOR THE CATABOLISM OF AROMATICS

A number of peripheral pathways for the catabolism of aromatic compounds have been identified by searching in the genome of *Pseudomonas* strains. The location and arrangement of the gene clusters encoding such peripheral pathways in the complete chromosome of *P. putida* KT2440 and *P. aeruginosa* PAO1 is indicated in Figure 2.

3.1. Pathways that Funnel to Protocatechuate

3.1.1. The 4-Hydroxybenzoate Pathway

4-Hydroxybenzoate is an abundant aromatic compound that is also generated during degradation of different aromatic hydrocarbons, insecticides, etc. (Figure 1). The *pobA* and *pobR* genes encode the monocomponent 4-hydroxybenzoate 3-monooxygenase (PobA), which hydroxylates 4-hydroxybenzoate to protocatechuate (Figure 3), and the cognate transcriptional activator (PobR)^{6, 20, 36, 101}. As observed in most bacteria, the *pobA* and *pobR* genes are divergently transcribed in *Pseudomonas* (Figure 4). Unlike PobR from *Acinetobacter* sp. ADP1 and some other bacteria, which belongs to the

IcIR family of transcriptional regulators, PobR from *Pseudomonas* strains shows similarity to regulators of the XylS/AraC family such as PobC from *P. putida* WCS358 that responds efficiently to 4-hydroxybenzoate and weakly to protocatechuate⁶. Although the *pob* genes are not linked to the *pca* genes in *P. putida*, *P. fluorescens* and *P. syringae*, they are associated in *P. aeruginosa* (Figure 4) and some other bacteria such as *Acinetobacter* sp. ADP1^{36, 45}.

3.1.2. The Quinate Pathway

Quinate is a very common hydroaromatic compound that, after initial degradation, becomes aromatic. The genes involved in quinate catabolism (*qui* cluster) are known in *Acinetobacter* sp. ADP1 and they encode the QuiA (quinate dehydrogenase), QuiB (type I dehydroquinate dehydratase) and QuiC (dehydroshikimate dehydratase) enzymes that transform this hydroaromatic compound into protocatechuate (Figure 3)¹⁹. The *qui* cluster from *Acinetobacter* sp. ADP1 contains also a putative porin-encoding gene (*quiX*) and it is located adjacent to the *pca* gene cluster^{67, 68}. Although *quiAX* homologs have been found in the genomes of *Pseudomonas* strains (PP3569 and PP3570 in *P. putida*; PA2290 and PA2291 in *P. aeruginosa*; Figure 2), no *quiBC* genes similar to those of *Acinetobacter* sp. ADP1 were identified. However, *quiB1* and *quiC1* genes encoding products that show similarity with the type II dehydroquinate dehydratase (QutE) and 3-dehydroshikimate dehydratase (QutC) from *Emericella nidulans*³⁷, are present in the genome of the *Pseudomonas* strains (PP3003 and PP2554 in *P. putida*; PA0245 and PA0244 in *P. aeruginosa*; Figures 2 and 4). All these data suggest that *quiB1* and *quiC1* genes might be involved in quinate metabolism in *Pseudomonas* (Figure 3), indicating that the quinate degradative enzymes in these bacteria might be different from those reported in *Acinetobacter*, which agrees with previous reports showing differences in quinate catabolism by these two genera⁴².

3.1.3. The Degradation Pathway of Phenylpropenoid Compounds

Phenylpropenoid compounds (e.g., cinnamate, ferulate, coumarate, etc.) form a vast array of ether and ester bonds in lignin and suberin⁶⁷, and these aromatic compounds constitute, therefore, a common carbon source for microorganisms that colonize the rhizosphere. In most bacteria, ferulic acid degradation follows a CoA-dependent non- β -oxidative pathway catalyzed by the Fcs (feruloyl-CoA synthetase) and Ech (enoyl-CoA hydratase/aldolase) proteins, producing vanillin (Figure 3)^{63, 73}. Vanillin is further converted to protocatechuate via an aldehyde dehydrogenase (*vdh* gene product) and a demethylase (*vanAB* gene products) (Figure 3)^{72, 82}. A cluster containing the *fcs*, *ech* and *vdh* genes, as well as a divergent regulatory gene (*ferR*) that encodes a protein of the MarR family, has been identified in *P. putida*, *P. fluorescens* and *P. syringae* (Figure 13)^{45, 70}. The *vanAB* orthologs have

been identified in all four *Pseudomonas* strains and they are clustered with a putative transcriptional repressor of the GntR family (*vanR*)⁵⁴ (Figure 13). The genes encoding a transporter (*vanK*) and a porin (*vanP*) in *Acinetobacter* sp. ADP1¹⁵ are also clustered with *vanABR* in *P. putida* (Figure 13). In *P. aeruginosa*, the *vanKABR* and *vanP* are separated by the *mdl* genes responsible of mandelate degradation (see Section 3.2.2; Figure 13). Although the same linkage of *mdlCD* and *van* genes was observed in *P. fluorescens* Pf-5, no *mdl* genes have been found yet in *P. fluorescens* Pf-01 (Figure 13). The location of the *fcs-vdh-ech* genes with respect to the *van* genes is different in the three *Pseudomonas* strains. Thus, whereas the *fcs-vdh-ech* cluster is not linked to the *van* genes in *P. putida* (Figures 2 and 13), these two clusters are separated by 35 kb in *P. syringae* and they constitute a single cluster in *P. fluorescens* (Figure 13). In other ferulate-degraders, for example, *P. putida* WCS358⁹⁸, *Pseudomonas* sp. HR199⁶³ and *Acinetobacter* sp. ADP1^{82, 84}, the *fcs/ech/vdh* genes (*hca* genes in *Acinetobacter*) form a cluster that is not linked to the *van* cluster. It was suggested that this gene organization would facilitate the appearance of spontaneous *van*-deficient strains in natural populations, which might allow the production of vanillate from ferulate as a chemical signal between plants and bacteria⁸².

Interestingly, two additional genes, *aat* (encoding a putative β -ketothiolase) and *acd* (encoding a putative acyl-CoA dehydrogenase) cluster with the *ech*, *vdh* and *fcs* genes (Figure 13), and they could be responsible of a CoA-dependent β -oxidative pathway for ferulic acid degradation that has been described in some organisms⁷³ (Figure 3). Moreover, an enzyme different from Vdh seems to be responsible for the oxidation of vanillin to vanillic acid during growth of *P. putida* KT2440 in the presence of vanillin, an observation that should be taken into account for the potential application of this strain as biocatalyst for vanillin production⁷⁰.

Catabolism of 4-coumaric acid and caffeic acid proceeds via 4-hydroxybenzoate and protocatechuate, respectively, through the action of the Fcs, Ech and Vdh enzymes (Figure 3)^{52, 98}. In *Acinetobacter* sp. ADP1, degradation of 4-hydroxyphenylpropionic and 3,4-dihydroxyphenylpropionic acids involves their dehydrogenation to 4-coumaroyl-CoA and caffeoyl-CoA, respectively, by the HcaD dehydrogenase⁸⁴. Putative *hcaD* genes have also been found in the genomes of the *Pseudomonas* strains. The catabolism of coniferyl alcohol in *Pseudomonas* sp. HR199 involves its conversion into ferulic acid by an alcohol dehydrogenase (CalA) and an aldehyde dehydrogenase (CalB)⁶³. Whereas a gene whose product shows sequence similarity with CalB from *Pseudomonas* sp. HR199 is found in the genome of *P. putida* (PP5120; Figure 2), *P. fluorescens* and *P. syringae*, a *calA* ortholog could only be identified in *P. syringae* and it is not linked to *calB*.

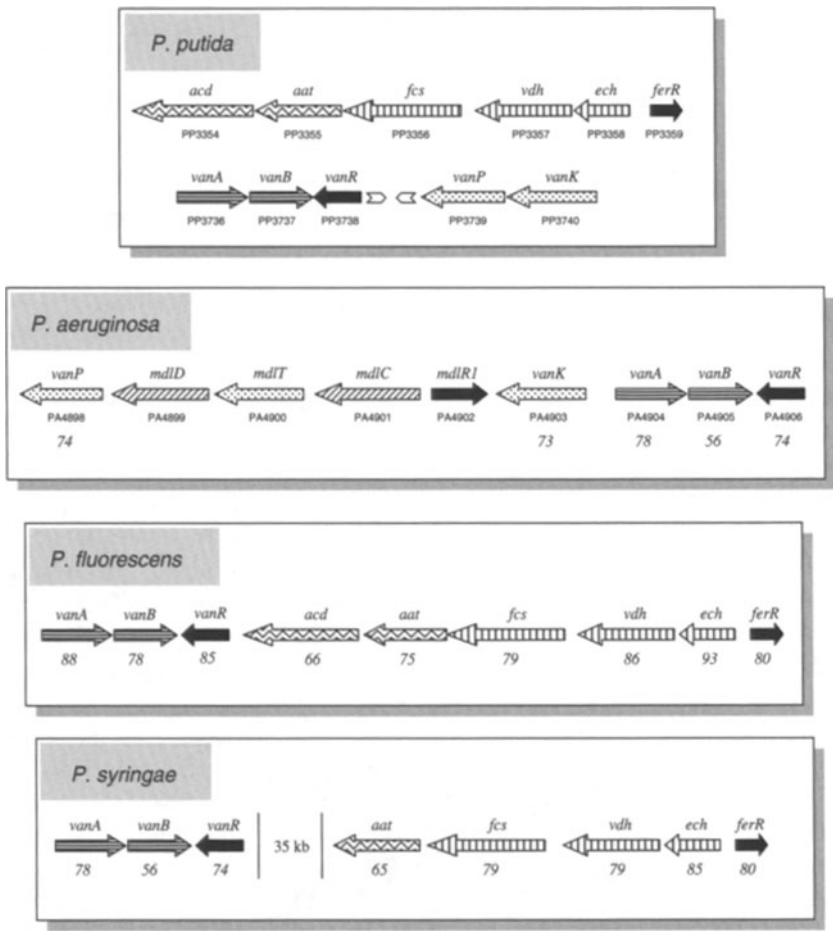


Figure 13. Gene arrangement of the *fcs/ech/vdh*, *van* and *mdl* clusters in *Pseudomonas*. Genes are represented by arrows: black (regulatory genes), stippled (transport genes), vertically striped (*ech*, *vdh*, and *fcs* genes), horizontally striped (genes encoding the VanAB vanillate-*O*-demethylase), hatched (*mdl* catabolic genes of the mandelate pathway), cross-hatched (*acd* and *aat* catabolic genes of the putative ferulate β -oxidative pathway). Arrowheads represent the *P. putida* REP element. PP and PA numbers below the genes indicate the corresponding proteins in the annotated genomes of *P. putida* and *P. aeruginosa*, respectively. Numbers underneath the arrows indicate the percentage of amino acid sequence identity between the encoded gene product and the equivalent product from *P. putida* KT2440.

3.2. Pathways that Funnel to Catechol

3.2.1. The Benzoate Pathway

Benzoate is an abundant aromatic acid and a key intermediate in the catabolism of a wide range of aromatic compounds (Figure 1). The *benABC* and *benD* genes encode the three-component benzoate dioxygenase and the benzoate dihydrodiol dehydrogenase, respectively, for the transformation of benzoate into catechol (Figure 5)^{9, 11, 44}. Although *ben* orthologs have been identified in the genomes of *P. putida*, *P. aeruginosa* and *P. fluorescens* (Figure 6), they are not present in the genome of *P. syringae*, which is in agreement with the lack of *cat* genes in this bacterium (see Section 2.1.2). Whereas the *ben* and *cat* clusters are distantly located in the genome of *P. putida* KT2440, they are contiguous in *P. fluorescens* (this species has two *ben-cat* clusters), and closely linked in *P. aeruginosa*¹⁰⁴ (Figures 2 and 6). The *ben* cluster from strain KT2440 shows two additional features that are not observed in *ben* clusters from other *Pseudomonas* strains: (a) it contains a second copy of the *cata* gene (*cata2*) whose product shows significant identity (77%) with the CatA dioxygenase of the catechol branch, (b) a gene (*benX*) of unknown function has been inserted between the *benR* and *benA* genes (Figure 6). While the *benE* gene encodes a membrane protein of unknown function, the *benK* and *benF* genes are likely to encode a benzoate transporter and a porin, respectively¹¹, and they are absent in *P. aeruginosa* (Figure 6). *benR* (Figure 6) is the ortholog of the gene encoding the transcriptional activator of the *ben* cluster that responds to benzoate in *P. putida* PRS2000¹¹. Whereas the expression of the *ben* genes in *Pseudomonas* is controlled by the *benR* gene product, which belongs to the XylS/AraC family, expression of the *ben* genes in *Acinetobacter* is controlled by BenM, a member of the LysR family of regulatory proteins⁹. The *benABCD* gene order was maintained in most clusters analyzed, being the *benR* and *benM* regulatory genes transcribed in the same and in the opposite direction than the catabolic genes, respectively⁴⁵.

A peripheral pathway that oxidizes benzylamine to benzoate should be expected in some *Pseudomonas* strains (Figure 5)⁸⁹, but the corresponding genes have not been yet characterized. In *P. putida* the synthesis of an inducible benzylamine dehydrogenase depends on the RNA polymerase sigma factor σ^{54} and is subject to carbon source-dependent inhibition⁴⁹.

3.2.2. The Mandelate Pathway

In *P. putida* ATCC 12633, the *mdl* gene cluster encodes the mandelate racemase (MdlA) that transforms D-mandelate to L-mandelate, the mandelate dehydrogenase (MdlB) that converts L-mandelate into benzoylformate, the benzoylformate decarboxylase (MdlC) that produces benzaldehyde, and

the benzaldehyde dehydrogenase (MdlD) that transforms the latter to benzoate (Figure 5). Moreover, an *mdlX* gene, encoding a putative regulatory protein, and a *mdlY* gene, encoding a mandelamide hydrolase, are also part of the *mdl* cluster in strain ATCC 12633⁵⁰. *P. aeruginosa* is also able to grow on L-mandelate as the sole carbon source using the pathway described above⁷⁸. The physiological and genetic properties of wild-type *P. aeruginosa* and various mutant strains lacking some enzymatic activities revealed that whereas MdlB is induced by L-mandelate, MdlC and MdlD are both induced by benzoylformate, being the cognate genes, *mdlC* and *mdlD*, closely linked in the genome^{78, 79}. In agreement with this, analysis of the *P. aeruginosa* chromosome revealed the existence of *mdl* orthologs arranged in two clusters, *mdlCTD* and *mdlB*, that are located at different chromosomal regions (Figures 2 and 13). The *mdlCTD* cluster contains the catabolic *mdlC* and *mdlD* genes and a gene encoding a putative transport protein (*mdlT*) (Figure 13). Although putative regulatory genes, *mdlR1* and *mdlR2*, are present in the *mdlCTD* and *mdlB* clusters, respectively (Figures 2 and 13), their products belong to the LysR family of transcriptional regulators and, therefore, they do not show similarity to the regulatory MdlX protein from *P. putida* ATCC12633. As expected, no *mdlA* ortholog was found in the genome of *P. aeruginosa*, which is in agreement with the observation that this strain does not use D-mandelate as carbon source⁷⁸.

Since *P. aeruginosa* uses vanillylmandelic acid as carbon source¹³, the MdlB and MdlC proteins could be also responsible for the transformation of this aromatic into vanillin⁹⁵ (Figure 3), which might explain the association of the *van* and *mdlCTD* genes in the genome of this strain (Figure 13).

3.2.3. The Anthranilate Pathway

Anthranilate is formed during the catabolism of tryptophan and some nitroaromatic compounds. The *antA*, *antB* and *antC* genes encode the large subunit, the small subunit and the reductase component, respectively, of the multicomponent anthranilate dioxygenase that converts anthranilate into catechol (Figure 5) in *Acinetobacter* sp. ADP1⁷. A homologous *ant* cluster is found in the genome of *P. aeruginosa* flanked by the *cat* and *ben* clusters (Figure 6). *P. aeruginosa ant*⁻ mutants have been described, and the linkage between *ant*, *cat* and *ben* genes had been previously reported^{79, 104}. The AntABC anthranilate dioxygenase is homologous to the BenABC benzoate dioxygenase (see Section 3.2.1). A putative regulatory gene, *antR*, whose product shows similarity to XylS/AraC regulators, is transcribed divergently to the catabolic *antABC* genes (Figure 6). The *cat-ant-ben* genes in *P. aeruginosa* are both functionally and physically associated and, therefore, they constitute an example of supraoperonic clustering (catabolic island) in *Pseudomonas*.

P. aeruginosa is also able to use L-tryptophan as a growth substrate⁸⁹. Tryptophan degradation in *Pseudomonas* usually involves formation of anthranilate via L-kynurenine (Figure 5)⁶². The genes encoding a putative tryptophan 2,3-dioxygenase (*tdo*, PA2579), formylkynurenine formamidase (*kynA*, PA2081) and kynureninase (*kynB*, PA2080) were identified in the genome of *P. aeruginosa* (Figure 2).

3.3. Pathways that Funnel to Phenylacetyl-CoA

The term catabolon defines a complex functional unit integrated by different catabolic pathways that catalyse the transformation of structurally related compounds into a common catabolite⁴⁸. The phenylacetyl-CoA catabolon in *P. putida* encompasses the routes involved in the transformation of 2-phenylethylamine, phenylacetic acid and *n*-phenylalkanoic acids containing an even number of carbon atoms into phenylacetyl-CoA (Figure 7)^{45, 48}.

Phenylethylamine is converted into phenylacetic acid via phenylacetaldehyde in different microorganisms. Although in most cases an aromatic amine oxidase (Mao) converts phenylethylamine into phenylacetaldehyde^{16, 31}, some *Pseudomonas* strains convert aromatic biogenic amines into the corresponding aromatic aldehydes via an amine dehydrogenase^{13, 17, 43}. Since no *mao* homolog was found in the genome of the *Pseudomonas* strains analyzed, it is likely that in these bacteria the catabolism of aromatic biogenic amines is carried out by the action of an amine dehydrogenase. The putative gene(s) encoding such aromatic amine dehydrogenase has not been yet identified. Phenylacetaldehyde is then oxidized to phenylacetic acid by a phenylacetaldehyde dehydrogenase (Pad) enzyme¹⁶ (Figure 7). Although there are several genes in the chromosome of *Pseudomonas* that show similarity to aryl aldehyde dehydrogenase-encoding genes, the gene encoding PP3646 from *P. putida* shows the highest identity to *pad* genes from other bacteria and it might correspond to the phenylacetaldehyde dehydrogenase from this bacterium (Figure 2).

Degradation of *n*-phenylalkanoic acids in *P. putida* requires their activation to CoA thioesters by an acyl-CoA synthetase encoded by *fadD*. Subsequently, an acyl-CoA dehydrogenase (*fadF* gene product) and a protein complex (FadAB) with five enzymatic activities catalyze, through β -oxidation, the release of acetyl-CoA units (Figure 7)⁶⁰. While the catabolism of phenylalkanoates containing an even number of carbon atoms produces phenylacetyl-CoA, degradation of phenylalkanoates with an odd number of carbon atoms produces *trans*-cinnamoyl-CoA which cannot be further catabolized and is excreted as cinnamic acid⁶⁰. The catabolism of *n*-phenylalkanoic acids in *P. putida* is carried out by two sets of β -oxidation enzymes: The β_1 oxidation set (clusters *fadBA* and *fadD1D2*; Figure 2) is constitutive and catalyzes a very

efficient degradation; the β_{II} set (cluster *fadDxB2xAxFxB1x*; Figure 2) is only expressed when some of the genes encoding the β_I enzymes are mutated and it catabolizes *n*-phenylalkanoates with an acyl moiety longer than four carbons⁶⁰.

3.4. Pathways that Funnel to Homogentisate

The homogentisate pathway is the central route through which tyrosine and phenylalanine are mineralized in many bacteria (Figure 9). The genes responsible for the peripheral pathway of the catabolism of phenylalanine and tyrosine in *P. aeruginosa* are known⁸⁶, and orthologs were found in the genomes of *P. putida*, *P. fluorescens* and *P. syringae*. The *phh* cluster encodes the putative pterin-dependent phenylalanine hydroxylase (*phhA*) that converts phenylalanine into tyrosine, the 4a-carbinolamine dehydratase (*phhB*) involved in regeneration of the pterin cofactor and in the positive regulation of PhhA, the σ^{54} -dependent transcriptional activator (*phhR*) of the *phh* operon⁸⁵, and a potential transport protein (*phhT*), that is absent in *P. aeruginosa*, close to a gene (*aroP2*) encoding a general aromatic amino acid permease (Figure 10). In *P. aeruginosa* the *phhC* gene encodes a tyrosine aminotransferase that transforms tyrosine into 4-hydroxyphenylpyruvic acid and is essential for the catabolism of either tyrosine or phenylalanine³⁰. The putative *phhC* homolog in the other three *Pseudomonas* species is the *tyrB* gene (two copies, *tyrB1* and *tyrB2*, are present at different positions of the *P. putida* genome; Figures 2 and 10). Whereas the *phh* genes form a cluster in *P. aeruginosa* and *P. fluorescens*, the *tyrB* genes are not linked to the *phhRABT* cluster in *P. putida* and *P. syringae* (Figure 10).

The *hpd* gene encodes the putative 4-hydroxyphenylpyruvic acid dioxygenase that converts 4-hydroxyphenylpyruvate into homogentisate⁸³ (Figure 9). The role of the *hpd* gene product coupling the catabolism of aromatic amino acids with the homogentisate central pathway might explain the linkage of *hpd* with the homogentisate cluster, such as in *P. syringae*, or with the *phh* cluster, such as in *P. aeruginosa* (Figure 10).

3.5. Pathways that Funnel to Homoprotocatechuate

In some bacteria 4-hydroxyphenylacetic acid is hydroxylated to homoprotocatechuate by a two-component HpaBC flavin-dependent monooxygenase (Figure 12)¹⁶. *hpaBC* orthologs have been found in the genomes of *P. aeruginosa* and *P. fluorescens* and, in contrast to the arrangement found in other bacteria such as *E. coli*, these two genes are not adjacent to the *hpa* central cluster responsible for homoprotocatechuate degradation (Figure 8C)¹⁶.

However, the *hpaA* regulatory gene, that activates expression of the *hpaBC* genes and is physically associated with them in *E. coli*, has an ortholog in *Pseudomonas* that is linked to the *hpa* central cluster (Figure 8C). Therefore, the organization of the *hpa* upper and central clusters differs between enteric bacteria and *Pseudomonas* strains. Differences in *hpa* catabolic and/or regulatory genes could also explain the observation that 3-hydroxyphenylacetic acid is degraded via homoprotocatechuate in enteric bacteria but via homogentisate in some *Pseudomonas*^{10, 88}.

Although *P. aeruginosa* cannot degrade phenylethylamine because it lacks the phenylacetic acid degradation pathway, this bacterium is able to degrade tyramine and some other phenolic amines. These biogenic amines can be formed by hydrolysis of arylsulfate esters through the action of the AtsA arylsulfatase (PA0183)⁵. Tyramine and dopamine are catabolized via an amine dehydrogenase (tyramine dehydrogenase) and a hydroxyphenylacetaldehyde dehydrogenase that produce 4-hydroxyphenylacetate (from tyramine) and homoprotocatechuate (from dopamine)¹³ (Figure 12). Octopamine, synephrine and norepinephrine are also used as carbon sources by *P. aeruginosa* via the homoprotocatechuate pathway (Figure 12)¹³. *P. aeruginosa* mutants unable to catabolize tyramine and dopamine have been described^{12, 13}, but the genes involved in this peripheral pathway are still unknown.

4. EVOLUTIONARY CONSIDERATIONS AND GENERAL CONCLUSIONS

Genomic search in *P. putida*, *P. aeruginosa*, *P. fluorescens* and *P. syringae* strains revealed the existence of at least five different central pathways for the catabolism of aromatic compounds. Interestingly, the modified *ortho*-cleavage pathway (*clc* cluster) responsible of chlorocatechol degradation⁷⁷, the alkylcatechol *meta*-cleavage pathway (*xyl* cluster)^{32, 33} and the hydroquinone and hydroxyquinol central pathways were not identified in any of the analyzed chromosomes. Whereas the β -ketoadipate and homogentisate pathways are present in all four *Pseudomonas* strains, the phenylacetate pathway is only present in *P. putida*. In contrast, whereas the homoprotocatechuate and gentisate pathways are present in *P. aeruginosa*, they are absent in *P. putida* KT2440. This heterogeneity in the catabolism of aromatics increases when peripheral pathways are analyzed. Thus, whereas *P. aeruginosa* harbors the genes responsible for the conversion of benzoate, anthranilate, tryptophan and mandelate to catechol, *P. putida* KT2440 contains only the *ben* genes for benzoate degradation. On the other hand, out of the four *Pseudomonas* species analyzed, *P. aeruginosa* is the only one lacking the *fcs/ech/vdh* genes involved in degradation of phenylpropenoid compounds. Nevertheless, the ability to

degrade aromatic compounds is a strain-specific feature and, for instance, a number of *P. putida* strains containing chromosomal central and peripheral pathways that are absent in strain KT2440 have been previously described. Thus, *P. putida* U contains the homoprotocatechuate pathway⁶¹, *P. putida* ATCC 12633 harbors the mandelate pathway⁵⁰, and *P. putida* F1 contains a catechol *meta*-cleavage pathway¹⁰⁶. Moreover, the location of the catabolic genes in mobile genetic elements such as plasmids (e.g., pWW0 for toluene/xylenes degradation²; pP51 for trichlorobenzene degradation⁹⁷, pVI150 for phenol degradation⁷¹, NAH7 for naphthalene degradation¹⁰²), transposons (e.g., Tn5280 and Tn5542 for chlorobenzene and benzene degradation, respectively)⁹³ and chromosomal elements with intercellular mobility (the *clc* element for chlorocatechol degradation in *Pseudomonas* sp. B13⁷⁶; the *bph-sal* element for biphenyl degradation in *P. putida* KF715⁵⁸, the *hyb* element for chlorobenzoate degradation in *P. aeruginosa* JB2³⁸) increases further the transfer of the catabolic pathways among different strains and contributes to the high metabolic diversity within the genus *Pseudomonas*. The TOL pathway (*xyl* cluster) of *P. putida* mt-2 is among the best-studied examples of aromatic hydrocarbon degradation encoded by a plasmid. The TOL plasmid pWW0 from *P. putida* mt-2 is a 116.5 kb catabolic plasmid that has been recently sequenced²⁹ and it encodes all proteins necessary for bacterial utilization of toluene, *m*- and *p*-xylene, 3-ethyltoluene and 1,2,4-trimethylbenzene (pseudocumene), plus their alcohol, aldehyde and carboxylic acid derivatives, via a *meta*-cleavage pathway². Therefore, the acquisition of the TOL plasmid by *P. putida* KT2440 significantly increases the catabolic abilities of the resulting strain toward aromatic compounds.

The G+C content of the clusters involved in catabolism of aromatic compounds in *P. putida* KT2440 and *P. aeruginosa* PAO1 is close to the mean G+C content of the chromosome of *P. putida* (63%)⁵⁶ and *P. aeruginosa* (66%)⁸⁷. This suggests that these sets of genes have been imprisoned within the chromosome of these bacteria over a long period of evolution.

The distribution of the aromatic catabolic clusters along the *P. putida* KT2440 chromosome reveals that the region (about 2,400 kb) flanking the replication origin (position 0 kb) is almost devoid of genes related to the catabolism of aromatic compounds, being the region located between positions 3,000 and 4,500 kb, the one showing the majority of the aromatic catabolic clusters (Figure 2). This situation contrasts with that observed in *P. aeruginosa* PAO1 where genes responsible for the catabolism of aromatic compounds are located near the replication origin (position 0 kb), and the regions that span from positions 1,000 to 2,000 kb and from 3,000 to 4,500 kb lack aromatic catabolic clusters (Figure 2). Moreover, whereas in *P. putida* there is not a supraoperonic clustering of catabolic genes (catabolic island) that channel different aromatic compounds into a common central pathway, as reported

for the *pca-qui-pob-ppa* island (suberon) in *Acinetobacter* sp. ADP1⁶⁸, in *P. aeruginosa* the *cat-ant-ben* genes constitute an example of a catabolic island located near the middle of the genome (Figure 2). Out of the four *Pseudomonas* species whose genome has been analyzed, *P. putida* KT2440 shows the lowest level of linkage between genes involved in the same aromatic catabolic pathway: the *ben* and *cat* genes are not associated; the *pca* genes are arranged in three different clusters and none of them is associated with the *pob* cluster and; the *tyrB* and *hpd* genes are not linked to the *phh* genes (Figure 2).

Some catabolic clusters from *P. putida* KT2440 show the presence of a 35 bp repetitive extragenic palindromic (REP) sequence previously reported in *P. putida* strains and whose function has not yet been identified^{1, 41} (Figures 4, 6, 8A, 10 and 13). The location of the REP sequence associated with some of the aromatic catabolic clusters in *P. putida* is strain-specific. Thus, while the *pha* cluster from strain KT2440 contains a single REP sequence downstream of the *phaL* gene (Figure 8A), the *pha* cluster from *P. putida* U contains two convergent REP elements in the *phaI-phaJ* intergenic region. On the other hand, whereas the two inverted REP sequences upstream of the *benE* gene in *P. putida* KT2240 are not present in *P. putida* PRS2000, the latter contains a REP element at the 3'-end of the *catR* gene⁴¹ that is absent in the *cat* cluster of *P. putida* KT2440. A different *P. putida* strain (*P. putida* RB1) shows the *cat*-associated REP element in the *catB-catC* intergenic region⁴¹. Therefore, although the genes are highly conserved among different *P. putida* strains, REP sequences appear to contribute significantly to genomic diversity within this species. Moreover, near the 3'-end of the *ben* cluster from *P. putida* KT2440 there is a gene encoding a putative maturase related protein of group II introns that is identical to the one reported previously in the vicinity of catabolic genes involved in degradation of *p*-cresol in *P. alcaligenes*¹⁰³, suggesting the involvement of group II introns in the evolution of catabolic functions, much like other mobile genetic elements.

The uptake of the compound inside the cell and the inducible expression of the catabolic genes are important regulatory issues in the catabolism of aromatic compounds. Most of the predicted inner membrane transport proteins from the aromatic catabolic pathways of *Pseudomonas*, that is, PcaK and PcaT (protocatechuate), BenK (benzoate), VanK (vanillate), HmgT (homogentisate), GtdT (gentisate), HpaX (homoprotocatechuate), MdlT (mandelate) and PhhT (phenylalanine) belong to the MFS of transporters, being PhaJ (phenylacetate) and AroP2 (aromatic amino acids) members of solute:sodium symporter (SSS) and amino acid-polyamine-choline (APC) families, respectively⁸¹. Aromatic transporters can be accompanied by outer membrane porins such as PcaP, PhaK, QuiX, VanP, BenF and MdlP. Although aromatic compounds can enter the cells by passive diffusion when present at high concentrations, active transport increases the efficiency and rate of substrate acquisition in natural environments where these compounds are present at low

concentrations⁵⁷. Moreover, the aromatic transporters can be involved in the ability of bacteria with a motile life-style, such as *Pseudomonas* strains, to sense and swim toward the aromatic compounds (chemotaxis)^{35, 36, 65}.

The regulatory mechanisms that control the expression of the genes responsible of the catabolism of aromatic compounds appear to be highly diverse in *Pseudomonas* strains. Thus, a genomic search allowed us to predict the existence of transcriptional activators or repressors from the XylS/AraC family (BenR, PobR, AntR, HpaA), IclR family (PcaR, HmgR), LysR family (CatR, PcaQ, GtdR, MdlR), NtrC family (PhhR), MarR family (FerR, HpaR) and GntR family (VanR and PhaN). Moreover, the pathway-specific regulation will be subordinated to a more general control that adjusts the particular transcriptional output to the physiological status of the cell⁸.

Although *Pseudomonas* turns out to be a very useful model system to study biochemical, genetic, evolutionary and ecological aspects of the catabolism of aromatic compounds, our current knowledge about the overall catabolic versatility of *Pseudomonas* strains toward aromatic compounds may still be far from complete. Thus, analysis of the *P. putida* KT2440 genome has shown the presence of several genes that are likely to be involved in the degradation and/or transformation of aromatic compounds. At positions 4,441–4,454 kb of the KT2440 genome there is a 13 kb gene cluster (*nic*) (Figure 2) that contains genes showing similarity with those encoding proteins involved in the metabolism of N-heterocyclic aromatic compounds²⁴. In addition, a gene cluster (*pcm*) containing genes similar to those encoding the protocatechuate 4,5-dioxygenase (*pcmA*) and oxalocitramalate aldolase (*pcmE*) from *Arthrobacter keyseri*¹⁸ was located at positions 2,861–2,867 kb of the KT2440 genome (Figure 2). Whether such gene clusters are involved in the catabolism of N-heterocyclic aromatic compounds (cluster *nic*) and in a central pathway for degradation of aromatic compounds via a 4,5-*meta* cleavage of the aromatic ring (cluster *pcm*), is the subject of current study. Furthermore, the pathways for degradation of phenylacetate, quinate and aromatic amines are not yet well understood in *Pseudomonas*, and further work needs to be done to identify the genes and/or the enzymatic steps involved in such catabolic routes. To unravel the physiological meaning of the gentisate pathway in *P. aeruginosa* is also the matter of future research. A deeper understanding of the complete set of aromatic catabolic abilities of *Pseudomonas* strains will pave the way for the rational design of more efficient and broad range biocatalysts for many biotechnological applications.

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REFERENCES

1. Aranda-Olmedo, I., Tobes, R., Manzanera, M., Ramos, J.L., and Marqués, S., 2002, Species-specific repetitive extragenic palindromic (REP) sequences in *Pseudomonas putida*. *Nucleic Acids Res.*, 30:1826–1833.
2. Assinder, S.J. and Williams, P.A., 1990, The TOL plasmids: Determinants of the catabolism of toluene and the xylenes. *Adv. Microbiol. Physiol.*, 31:1–69.
3. Bagdasarian, M., Lurz, R., Rückert, B., Franklin, F.C.H., Bagdasarian, M.M., and Timmis, K.N., 1981, Specific-purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. *Gene*, 16:237–247.
4. Bayley, S.A., Duggleby, C.J., Worsey, M.J., Williams, P.A., Hardy, K.G., and Broda, P., 1977, Two modes of loss of the TOL function from *Pseudomonas putida* mt-2. *Mol. Gen. Genet.*, 154:203–204.
5. Beil, S., Kehrli, H., James, P., Staudenmann, W., Cook, A.M., Leisinger, T., and Kertesz, M.A., 1995, Purification and characterization of the arylsulfatase synthesized by *Pseudomonas aeruginosa* PAO during growth in sulfate-free medium and cloning of the arylsulfatase gene (*atsA*). *Eur. J. Biochem.*, 229:385–394.
6. Bertani, I., Kojic, M., and Venturi, V., 2001, Regulation of the *p*-hydroxybenzoic acid hydroxylase gene (*pobA*) in plant-growth-promoting *Pseudomonas putida* WCS358. *Microbiology*, 147:1611–1620.
7. Bundy, B.M., Campbell, A.L., and Neidle, E.L., 1998, Similarities between the *antABC*-encoded anthranilate dioxygenase and the *benABC*-encoded benzoate dioxygenase of *Acinetobacter* sp. strain ADP1. *J. Bacteriol.*, 180:4466–4474.
8. Cases, I. and de Lorenzo, V., 2001, The black cat/white cat principle of signal integration in bacterial promoters. *EMBO J.*, 20:1–11.
9. Collier, L.S., Gaines, G.L.I., and Neidle, E.L., 1998, Regulation of benzoate degradation in *Acinetobacter* sp. strain ADP1 by BenM, a LysR-type transcriptional activator. *J. Bacteriol.*, 180:2493–2501.
10. Cooper, R.A. and Skinner, M.A., 1980, Catabolism of 3- and 4-hydroxyphenylacetate by the 3,4-dihydroxyphenylacetate pathway in *Escherichia coli*. *J. Bacteriol.*, 143:302–306.
11. Cowles, C.E., Nichols, N.N., and Harwood, C.S., 2000, BenR, a XylS homologue, regulates three different pathways of aromatic acid degradation in *Pseudomonas putida*. *J. Bacteriol.*, 182:6339–6346.
12. Cuskey, S.M. and Olsen, R.H., 1988, Catabolism of aromatic biogenic amines by *Pseudomonas aeruginosa* PAO1 via *meta* cleavage of homoprotocatechuic acid. *J. Bacteriol.*, 170:393–399.
13. Cuskey, S.M., Peccoraro, V., and Olsen, R.H., 1987, Initial catabolism of aromatic biogenic amines by *Pseudomonas aeruginosa* PAO: Pathway description, mapping of mutations, and cloning of essential genes. *J. Bacteriol.*, 169:2398–2404.
14. Dagley, S., 1986, Biochemistry of aromatic hydrocarbon degradation in *Pseudomonads*. In J. Sokatch and J.L. Ornston (eds), *The Bacteria*, vol. 10, pp. 527–555. Academic Press Inc., Orlando, FL.

15. D'Argenio, D.A., Segura, A., Coco, W.M., Bünz, P.V., and Ornston, L.N., 1999, The physiological contribution of *Acinetobacter* PcaK, a transport system that acts upon protocatechuate, can be masked by the overlapping specificity of VanK. *J. Bacteriol.*, 181:3505–3515.
16. Diaz, E., Ferrández, A., Prieto, M.A., and García, J.L., 2001, Biodegradation of aromatic compounds by *Escherichia coli*. *Microbiol. Mol. Biol. Rev.*, 65:523–569.
17. Durham, D.R. and Perry, J.J., 1978, Purification and characterization of a heme-containing amine dehydrogenase from *Pseudomonas putida*. *J. Bacteriol.*, 134:837–843.
18. Eaton, R.W., 2001, Plasmid-encoded phthalate catabolic pathway in *Arthrobacter keyseri* 12B. *J. Bacteriol.*, 183:3689–3703.
19. Elsemore, D.A. and Ornston, L.N., 1995, Unusual ancestry of dehydratases associated with quinate catabolism in *Acinetobacter calcoaceticus*. *J. Bacteriol.*, 177:5971–5978.
20. Entsch, B., Nan, Y., Weaich, K., and Scott, K.F., 1988, Sequence and organization of *pobA*, the gene coding for *p*-hydroxybenzoate hydroxylase, an inducible enzyme from *Pseudomonas aeruginosa*. *Gene*, 71:279–291.
21. Eulberg, D., Lakner, S., Golovleva, L.A., and Schlömann, M., 1998, Characterization of a protocatechuate catabolic gene cluster from *Rhodococcus opacus* 1CP: Evidence for a merged enzyme with 4-carboxymuconolactone-decarboxylating and 3-oxoadipate enol-lactone-hydrolyzing activity. *J. Bacteriol.*, 180:1072–1081.
22. Fernández-Cañón, J. and Peñalva, M.A., 1998, Characterization of a fungal maleylacetoacetate isomerase gene and identification of its human homologue. *J. Biol. Chem.*, 273:329–337.
23. Ferrández, A., Miñambres, B., García, B., Olivera, E.R., Luengo, J.M., García, J.L., and Diaz, E., 1998, Catabolism of phenylacetic acid in *Escherichia coli*. Characterization of a new aerobic hybrid pathway. *J. Biol. Chem.*, 273:25974–25986.
24. Fetzner, S., 1998, Bacterial degradation of pyridine, indole, quinoline, and their derivatives under different redox conditions. *Appl. Microbiol. Biotechnol.*, 49:237–250.
25. Franklin, F.C.H., Bagdasarian, M., Bagdasarian, M.M., and Timmis, K.N., 1981, Molecular and functional analysis of the TOL plasmid pWW0 from *Pseudomonas putida* and cloning of genes for the entire regulated aromatic ring *meta* cleavage pathway. *Proc. Natl. Acad. Sci. USA*, 78:7458–7462.
26. Fukimori, F., Hirayama, H., Takami, H., Inoue, A., and Horikoshi, K., 1998, Isolation and transposon mutagenesis of a *Pseudomonas putida* KT2442 toluene-resistant variant: Involvement of an efflux system in solvent resistance. *Extremophiles*, 2:395–400.
27. García, B., Olivera, E.R., Miñambres, B., Carnicero, D., Muñoz, C., Naharro, G., and Luengo, J.M., 2000, Phenylacetyl-coenzyme A is the true inducer of the phenylacetic acid catabolism pathway in *Pseudomonas putida* U. *Appl. Environ. Microbiol.*, 66:4575–4578.
28. Gou, Z. and Houghton, J.E., 1999, PcaR-mediated activation and repression of *pca* genes from *Pseudomonas putida* are propagated by its binding to both the –35 and the –10 promoter elements. *Mol. Microbiol.*, 32:253–263.
29. Greated, A., Lambertsen, L., Williams, P.A., and Thomas, C.M., 2002, Complete sequence of the IncP-9 TOL plasmid pWW0 from *Pseudomonas putida*. *Environ. Microbiol.*, 4:856–871.
30. Gu, W., Song, J., Bonner, C.A., Xie, G., and Jensen, R.A., 1998, PhhC is an essential amino-transferase for aromatic amino acid catabolism in *Pseudomonas aeruginosa*. *Microbiology*, 144:3127–3134.
31. Hacısalihoglu, A., Jongejan, J.A., and Duine, J.A., 1997, Distribution of amine oxidases and amine dehydrogenases in bacteria grown on primary amines and characterization of the amine oxidase from *Klebsiella oxytoca*. *Microbiology*, 143:505–512.
32. Harayama, S. and Timmis, K.N., 1989, Catabolism of aromatic hydrocarbons by *Pseudomonas*. In A. Hopwood and K.F. Chater (eds), *Genetics of bacterial diversity*. Academic Press, London.

33. Harayama, S. and Timmis, K.N., 1992, Aerobic biodegradation of aromatic hydrocarbons by bacteria. In H. Sigel and A. Sigel (eds), *Metal Ions in Biological Systems*. Marcel Dekker, Inc., New York, NY.
34. Harwood, C.S., Burchhardt, G., Herrmann, H., and Fuchs, G., 1999, Anaerobic metabolism of aromatic compounds via the benzoyl-CoA pathway. *FEMS Microbiol. Rev.*, 22:439–458.
35. Harwood, C.S., Nichols, N.N., Kim, M.-K., Ditty, J.L., and Parales, R.E., 1994, Identification of the *pcaRKF* gene cluster from *Pseudomonas putida*: Involvement in chemotaxis, biodegradation, and transport of 4-hydroxybenzoate. *J. Bacteriol.*, 176:6479–6488.
36. Harwood, C.S. and Parales, R.E., 1996, The β -ketoadipate pathway and the biology of self-identity. *Annu. Rev. Microbiol.*, 50:553–590.
37. Hawkins, A.R., Lamb, H.K., Smith, M., Keyte, J.W., and Roberts, C.F., 1988, Molecular organisation of the quinic acid utilization (*QUT*) gene cluster in *Aspergillus nidulans*. *Mol. Gen. Genet.*, 214:224–231.
38. Hickey, W.J., Sabat, G., Yuroff, A.S., Arment, A.R., and Pérez-Lesher, J., 2001, Cloning, nucleotide sequencing, and functional analysis of a novel, mobile cluster of biodegradation genes from *Pseudomonas aeruginosa* strain JB2. *Appl. Environ. Microbiol.*, 67:4603–4609.
39. Hirano, S.S. and Upper, C.D., 2000, Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae*—a pathogen, ice nucleus and epiphyte. *Microbiol. Mol. Biol. Rev.*, 64:624–653.
40. Hoet, P.P. and Stanier, R.Y., 1970, Existence and functions of the two enzymes with β -ketoadipate:Succinyl-CoA transferase activity in *Pseudomonas fluorescens*. *Eur. J. Biochem.*, 13:71–76.
41. Houghton, J.E., Brown, T.M., Appel, A.J., Hughes, E.J., and Ornston, L.N., 1995, Discontinuities in the evolution of *Pseudomonas putida cat* genes. *J. Bacteriol.*, 177:401–412.
42. Ingledew, W.M. and Tai, C.C., 1972, Quinate metabolism in *Pseudomonas aeruginosa*. *Can. J. Microbiol.*, 18:1817–1824.
43. Iwaki, M., Yagi, T., Horiike, K., Saeki, Y., Ushijima, T., and Nozaki, M., 1983, Crystallization and properties of aromatic amine dehydrogenase from *Pseudomonas* sp. *Arch. Biochem. Biophys.*, 220:253–262.
44. Jeffrey, W.H., Cuskey, S.M., Chapman, P.J., Resnick, S., and Olsen, R.H., 1992, Characterization of *Pseudomonas putida* mutants unable to catabolize benzoate: Cloning and characterization of *Pseudomonas* genes involved in benzoate catabolism and isolation of a chromosomal DNA fragment able to substitute for *xylS* in activation of the TOL lower-pathway promoter. *J. Bacteriol.*, 174:4986–4996.
45. Jiménez, J.I., Miñambres, B., García, J.L., and Díaz, E., 2002, Genomic analysis of the aromatic catabolic pathways from *Pseudomonas putida* KT2440. *Environ. Microbiol.*, 4:824–841.
46. Knackmuss, H.-J., 1996, Basic knowledge and perspectives of bioelimination of xenobiotic compounds. *J. Biotechnol.*, 51:287–295.
47. Kukor, J.J., Olsen, R.H., and Ballou, D.P., 1988, Cloning and expression of the *catA* and *catBC* gene clusters from *Pseudomonas aeruginosa* PAO. *J. Bacteriol.*, 170:4458–4465.
48. Luengo, J.M., García, J.L., and Olivera, E.R., 2001, The phenylacetyl-CoA catabolon: A complex catabolic unit with broad biotechnological applications. *Mol. Microbiol.*, 39:1434–1442.
49. Mallavarapu, M., Möhler, I., Krüger, M., Hosseini, M., Bartels, F., Timmis, K.N., and Holtel, A., 1998, Genetic requirements for the expression of benzylamine dehydrogenase activity in *Pseudomonas putida*. *FEMS Microbiol. Lett.*, 166:109–114.
50. McLeish, M.J., Kneen, M.M., Gopalakrishna, K.N., Koo, C.W., Babbitt, P.C., Gerlt, J.A., and Kenyon, G.L., 2003, Identification and characterization of a mandelamide hydrolase and an

- NAD(P)⁺-dependent benzaldehyde dehydrogenase from *Pseudomonas putida* ATCC 12633. *J. Bacteriol.*, 185:2451–2456.
51. Milcamps, A. and de Bruijn, F.J., 1999, Identification of a novel nutrient-deprivation-induced *Sinorhizobium meliloti* gene (*hmgA*) involved in the degradation of tyrosine. *Microbiology*, 145:935–947.
 52. Mitra, A., Kitamura, Y., Gasson, M.J., Narbad, A., Parr, A.J., Payne, J., Rhodes, M.J.C., Sewter, C., and Walton, N.J., 1999, 4-Hydroxycinnamoyl-CoA hydratase/lyase (HCHL)—an enzyme of phenylpropanoid chain cleavage from *Pseudomonas*. *Arch. Biochem. Biophys.*, 365:10–16.
 53. Mohamed, M.E., Ismail, W., Heider, J., and Fuchs, G., 2002, Aerobic metabolism of phenylacetic acids in *Azoarcus evansii*. *Arch. Microbiol.*, 178:180–192.
 54. Morawski, B., Segura, A., and Ornston, L.N., 2000, Repression of *Acinetobacter* vanillate demethylase synthesis by VanR, a member of the GntR family of transcriptional regulators. *FEMS Microbiol. Lett.*, 187:65–68.
 55. Nakai, C., Horiike, K., Kuramitsu, S., Kagamiyama, H., and Nozaki, M., 1990, Three isoenzymes of catechol 1,2-dioxygenase (pyrocatechase), $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$, from *Pseudomonas arvilla* C-1. *J. Biol. Chem.*, 265:660–665.
 56. Nelson, K.E., Weinell, C., Paulsen, I.T., Dodson, R.J., Hilbert, H., Martins dos Santos, V.A.P., Fouts, D.E., Gill, S.R., Pop, M., Holmes, M., Brinkac, L., Beanan, M., DeBoy, R.T., Daugherty, S., Kolonay, J., Madupu, R., Nelson, W., White, O., Peterson, J., Khouri, H., Hance, I., Chris Lee, P., Holtzapple, E., Scanlan, D., Tran, K., Moazzzez, A., Utterback, T., Rizzo, M., Lee, K., Kosack, D., Moestl, D., Wedler, H., Lauber, J., Stjepandic, D., Hoheisel, J., Straetz, M., Heim, S., Kiewitz, C., Eisen, J., Timmis, K.N., Dusterhöft, A., Tümmeler, B., and Fraser, C.M., 2002, Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ. Microbiol.*, 4:799–808.
 57. Nichols, N.N. and Harwood, C.S., 1997, PcaK, a high-affinity permease for the aromatic compounds 4-hydroxybenzoate and protocatechuate from *Pseudomonas putida*. *J. Bacteriol.*, 179:5056–5061.
 58. Nishi, A., Tominaga, K., and Furukawa, K., 2000, A 90-kilobase conjugative chromosomal element coding for biphenyl and salicylate catabolism in *Pseudomonas putida* KF715. *J. Bacteriol.*, 182:1949–1955.
 59. Nozaki, M., Kagamiyama, H., and Hayaishi, O., 1963, Crystallization and some properties of metapyrocatechase. *Biochem. Biophys. Res. Commun.*, 11:65–70.
 60. Olivera, E.R., Carnicero, D., García, B., Miñambres, B., Moreno M.A., Cañedo, L., DiRusso, C.C., Naharro, G., and Luengo, J.M., 2001, Two different pathways are involved in the β -oxidation of *n*-alkanoic and *n*-phenylalkanoic acids in *Pseudomonas putida* U: Genetic studies and biotechnological applications. *Mol. Microbiol.*, 39:863–874.
 61. Olivera, E.R., Reglero, A., Martínez-Blanco, H., Fernández-Medarde, A., Moreno, M.A., and Luengo, J.M., 1994, Catabolism of aromatics in *Pseudomonas putida* U. Formal evidence that phenylacetic acid and 4-hydroxyphenylacetic acid are catabolized by two unrelated pathways. *Eur. J. Biochem.*, 221:375–381.
 62. Ornston, L.N., 1971, Regulation of catabolic pathways in *Pseudomonas*. *Bacteriol. Rev.*, 35:87–116.
 63. Overhage, J., Priefert, H., and Steinbüchel, A., 1999, Biochemical and genetic analyses of ferulic acid catabolism in *Pseudomonas* sp. strain HR199. *Appl. Environ. Microbiol.*, 65:4837–4847.
 64. Palleroni, N.J., 2003, Prokaryote taxonomy of the 20th century and the impact of studies on the genus *Pseudomonas*: A personal view. *Microbiology*, 149:1–7.
 65. Parales, R.E. and Harwood, C.S., 2002, Bacterial chemotaxis to pollutants and plant-derived aromatic molecules. *Curr. Opin. Microbiol.*, 5:266–273.

66. Parke, D., 1996, Characterization of PcaQ, a LysR-type transcriptional activator required for catabolism of phenolic compounds, from *Agrobacterium tumefaciens*. *J. Bacteriol.*, 178:266–272.
67. Parke, D., D'Argenio, D.A., and Ornston, L.N., 2000, Bacteria are not what they eat: That is why they are so diverse. *J. Bacteriol.*, 182:257–263.
68. Parke, D., García, M.A., and Ornston, L.N., 2001, Cloning and genetic characterization of *dca* genes required for β -oxidation of straight-chain dicarboxylic acids in *Acinetobacter* sp. strain ADP1. *Appl. Environ. Microbiol.*, 67:4817–4827.
69. Pieper, D.H. and Reineke, W., 2000, Engineering bacteria for bioremediation. *Curr. Opin. Biotechnol.*, 11:262–270.
70. Plaggenborg, R., Overhage, J., Steinbüchel, A., and Priefert, H., 2003, Functional analyses of genes involved in the metabolism of ferulic acid in *Pseudomonas putida* KT2440. *Appl. Microbiol. Biotechnol.*, 61:528–535.
71. Powlowski, J. and Shingler, V., 1994, Genetics and biochemistry of phenol degradation by *Pseudomonas* sp. CF600. *Biodegradation*, 5:219–236.
72. Priefert, H., Rabenhorst, J., and Steinbüchel, A., 1997, Molecular characterization of genes of *Pseudomonas* sp. strain HR199 involved in bioconversion of vanillin to protocatechuate. *J. Bacteriol.*, 179:2595–2607.
73. Priefert, H., Rabenhorst, J., and Steinbüchel, A., 2001, Biotechnological production of vanillin. *Appl. Microbiol. Biotechnol.*, 56:296–314.
74. Ramos, J.L., Díaz, E., Dowling, D., de Lorenzo, V., Molin, S., O'Gara, F., Ramos, C., and Timmis, K.N., 1994, The behaviour of bacteria designed for biodegradation. *Biotechnology*, 12:1349–1356.
75. Ramos, J.L., Duque, E., Godoy, P., and Segura, A., 1998, Efflux pumps involved in toluene tolerance in *Pseudomonas putida* DOT-T1E. *J. Bacteriol.*, 180:3323–3329.
76. Ravatn, R., Studer, S., Springael, D., Zehnder, A.J.B., van der Meer, J.R., 1998, Chromosomal integration, tandem amplification, and deamplification in *Pseudomonas putida* F1 of a 105-kilobase genetic element containing the chlorocatechol degradative genes from *Pseudomonas* sp. strain B13. *J. Bacteriol.*, 180:4360–4369.
77. Reineke, W., 1998, Development of hybrid strains for the mineralization of chloroaromatics by patchwork assembly. *Annu. Rev. Microbiol.*, 52:287–331.
78. Rosenberg, S.L., 1971, Regulation of the mandelate pathway in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 108:1257–1269.
79. Rosenberg, S.L. and Hegeman, G.D., 1971, Genetics of the mandelate pathway in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 108:1270–1276.
80. Rothmel, R.K., Aldrich, T.L., Houghton, J.E., Coco, W.M., Ornston, L.N., and Chakrabarty, A.M., 1990, Nucleotide sequencing and characterization of *Pseudomonas putida* *catR*: A positive regulator of the *catBC* operon is a member of the LysR family. *J. Bacteriol.*, 172:922–931.
81. Saier, M.H., Jr, 1998, Molecular phylogeny as a basis for the classification of transport proteins from bacteria, archaea, and eukarya. *Adv. Microb. Physiol.*, 40:81–136.
82. Segura, A., Bünz, P.V., D'Argenio, D.A., and Ornston, L.N., 1999, Genetic analysis of a chromosomal region containing *vanA* and *vanB*, genes required for conversion of either ferulate or vanillate to protocatechuate in *Acinetobacter*. *J. Bacteriol.*, 181:3494–3504.
83. Serre, L., Sailland, A., Sy, D., Boudec, P., Rolland, A., Pebay-Peyroula, E., and Cohen-Addad, C., 1999, Crystal structure of *Pseudomonas fluorescens* 4-hydroxyphenylpyruvate dioxygenase: An enzyme involved in the tyrosine degradation pathway. *Structure*, 7:977–988.
84. Smith, M.A., Weaver, V.B., Young, D.M., and Ornston, L.N., 2003, Genes for chlorogenate and hydroxycinnamate catabolism (*hca*) are linked to functionally related genes in the *dca-pca-qui-pob-hca* chromosomal cluster of *Acinetobacter* sp. strain ADP1. *Appl. Environ. Microbiol.*, 69:524–532.

85. Song, J. and Jensen, R.A., 1996, PhhR, a divergently transcribed activator of the phenylalanine hydroxylase gene cluster of *Pseudomonas aeruginosa*. *Mol. Microbiol.*, 22:497–507.
86. Song, J., Xia, T., and Jensen, R.A., 1999, PhhB, a *Pseudomonas aeruginosa* homolog of mammalian pterin 4a-carbinolamine dehydratase/DcoH, does not regulate expression of phenylalanine hydroxylase at the transcriptional level. *J. Bacteriol.*, 181:2789–2796.
87. Spain, J.C., 1995, Biodegradation of nitroaromatic compounds. *Annu. Rev. Microbiol.*, 49:523–555.
88. Sparrins, V.L., Chapman, P.J., and Dagley, S., 1974, Bacterial degradation of 4-hydroxyphenylacetic acid and homoprotocatechuic acid. *J. Bacteriol.*, 120:159–167.
89. Stanier, R.Y., Palleroni, N.J., and Doudoroff, M., 1966, The aerobic pseudomonads: A taxonomic study. *J. Gen. Microbiol.*, 43:159–271.
90. Spiers, A.J., Buckling, A., and Rainey, P.B., 2000, The causes of *Pseudomonas* diversity. *Microbiology*, 146:2345–2350.
91. Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrenner, P., Hickey, M.J., Brinkman, F.S.L., Hufnagle, W.O., Kowalik, D.J., Lagrou, M., Garber, R.L., Goltry, L., Tolentino, E., Westbrook-Wadman, S., Yuan, Y., Brody, L.L., Coulter, S.N., Folger, K.R., Kas, A., Larbig, K., Lim, L., Smith, K., Spencer, D., Wong, G.K.-S., Wu, Z., Paulsen, I.T., Reizer, J., Saier, M.H., Hancock, R.E.W., Lory, S., and Olson, M.V., 2000, Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature*, 406:959–964.
92. Suzuki, K., Ichimura, A., Ogawa, N., Hasebe, A., and Miyashita, K., 2002, Differential expression of two catechol 1,2-dioxygenases in *Burkholderia* sp. strain TH2. *J. Bacteriol.*, 184:5714–5722.
93. Tan, H.-M., 1999, Bacterial catabolic transposons. *Appl. Microbiol. Biotechnol.*, 51:1–12.
94. Timmis, K.N., 2002, *Pseudomonas putida*: A cosmopolitan opportunist *par excellence*. *Environ. Microbiol.*, 4:779–781.
95. Turner, J.E., Allison, N., and Fewson, C.A., 1996, Metabolic characterisation of a novel vanillylmandelate-degrading bacterium. *Arch. Microbiol.*, 166:252–259.
96. van der Meer, J.R., de Vos, W.M., Harayama, S., and Zehnder, A.J.B., 1992, Molecular mechanisms of genetic adaptation to xenobiotic compounds. *Microbiol. Rev.*, 56:677–694.
97. van der Meer, J.R., van Neerven, A.R.W., de Vries, E.J., de Vos, W.M., and Zehnder, A.J.B., 1991, Cloning and characterization of plasmid-encoded genes for the degradation of 1,2-dichloro-, 1,4-dichloro-, and 1,2,4-trichlorobenzene of *Pseudomonas* sp. strain P51. *J. Bacteriol.*, 173:6–15.
98. Venturi, V., Zennaro, F., Degrassi, G., Okeke, B.C., and Bruschi, C.V., 1998, Genetics of ferulic acid bioconversion to protocatechuic acid in plant-growth-promoting *Pseudomonas putida* WCS358. *Microbiology*, 144:965–973.
99. Wackett, L.P., 2003, *Pseudomonas putida*—a versatile biocatalyst. *Nat. Biotechnol.*, 21:136–138.
100. Walsh, U.F., Morrissey, J.P., and O’Gara, F., 2001, *Pseudomonas* for biocontrol of phytopathogens: From functional genomics to commercial exploitation. *Curr. Opin. Biotechnol.*, 12:289–295.
101. Wang, J., Ortiz-Maldonado, M., Entsch, B., Massey, V., Ballou, D., and Gatti, D.L., 2002, Protein and ligand dynamics in 4-hydroxybenzoate hydroxylase. *Proc. Natl. Acad. Sci. USA*, 99:608–613.
102. Yen, K.-M. and Serdar, C.M., 1988, Genetics of naphthalene catabolism in pseudomonads. *Crit. Rev. Microbiol.*, 15:247–268.
103. Yeo, C.C., Tham, J.M., Yap, M.W.-C., and Poh, C.L., 1997, Group II intron from *Pseudomonas alcaligenes* NCIB 9867 (P25X): Entrapment in plasmid RP4 and sequence analysis. *Microbiology*, 143:2833–2840.

104. Zhang, C., Huang, M., and Holloway, B.W., 1993, Mapping of the *ben*, *ant* and *cat* genes of *Pseudomonas aeruginosa* and evolutionary relationship of the *ben* region of *P. aeruginosa* and *P. putida*. *FEMS Microbiol. Lett.*, 108:303–310.
105. Zhou, N.-Y., Fuenmayor, S.L., and Williams, P.A., 2001, *nag* genes of *Ralstonia* (formerly *Pseudomonas*) sp. strain U2 encoding enzymes for gentisate catabolism. *J. Bacteriol.*, 183:700–708.
106. Zylstra, G.J. and Gibson, D.T., 1989, Toluene degradation by *Pseudomonas putida* F1: Nucleotide sequence of the *todC1C2BADE* genes and their expression in *Escherichia coli*. *J. Biol. Chem.*, 264:14940–14946.

CATABOLISM OF PAHs

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1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) refer to hydrocarbons containing two or more fused benzene rings in a linear, angular, and cluster arrangement (Figure 1). PAHs are mainly formed as products from the combustion of fossil fuels, as by-products of industrial processing and during the cooking of foods⁹¹. PAHs enter the environment from a multiplicity of sources which include direct aerial fallout, chronic leakage of industrial or sewage effluent, accidental discharge during transport, use and disposal of petroleum products, and from natural sources such as oil seepage and surface water run-off from forest and prairie fire sites. More specifically, industrial effluent from coal gasification and liquefaction processes, waste incineration, coke, carbon black, and other petroleum-derived products releases high quantities of PAHs into the environment. PAH contamination, particularly from the high-molecular-weight types, in soil and aquifers is a cause of great environmental concern because of their toxic, mutagenic, and carcinogenic effects on experimental animals and their potential health risk to humans^{19, 38, 95}. Some PAHs are classified as priority pollutants to be monitored in aquatic and terrestrial ecosystems by the U.S.

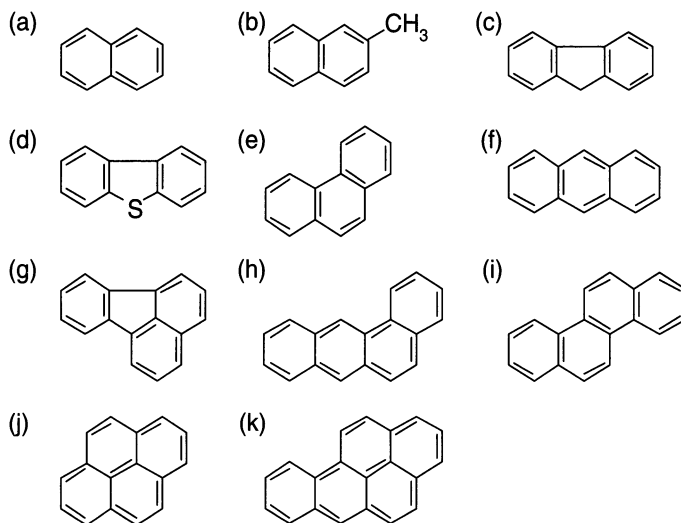


Figure 1. Structures of some aromatic hydrocarbons that have been detected in environmental samples: (a) naphthalene, (b) 2-methylnaphthalene, (c) fluorine, (d) dibenzothiophene, (e) phenanthrene, (f) anthracene, (g) fluoranthene, (h) benz[a]anthracene, (i) chrysene, (j) pyrene, (k) benzo[a]pyrene.

Environmental Protection Agency^{75, 125}, and benzo[a]pyrene is included as one of twelve target compounds or groups defined in the Environmental Protection Agency's new strategy for controlling persistent, bioaccumulative, and toxic pollutants¹¹⁰. Table 1 lists the physical and toxicological characteristics of certain PAHs.

PAHs are hydrophobic compounds, and their persistence within an ecosystem is due to their low solubility in water. PAHs rapidly become associated with sediment^{50, 58, 59, 97} where they may become buried and persist until degraded, resuspended, bioaccumulated, or removed by dredging. The environmental persistence of PAHs is correlated with their molecular size, and inversely correlated with the environmental biodegradation rate^{6, 13, 55, 57}. For example, the reported half-life in soil and sediment of the naphthalene molecule may range from 17 to 31 days, while for the five-ring molecule, benzo[a]pyrene, may range from 229 to >1,400 days¹²¹. In addition to the increased environmental persistence with increasing PAH molecular size, evidence suggests that, in some cases, PAH genotoxicity also increases with size up to at least four or five fused benzene rings¹⁹.

Studies on the environmental distribution of PAHs were initiated in 1947 when Kern reported the presence of chrysene in soil samples⁷⁹. The advent of new and improved analytical techniques such as gas chromatography and mass spectrometry and of improved methods for the extraction and isolation of PAHs

Table 1. Physical and toxicological^a properties of selected PAHs.

PAH	MW	Solubility in water (mg/l)	Half-life ^b (weeks)	Carcinogenicity	Genotoxicity
Naphthalene	128.2	31.7	0.02–46	—	—
Acenaphthene	154.2	3.9	0.04–0.6	—	+ (Ames)
Anthracene	178.2	0.7	0.5–26	—	—
Phenanthrene	178.2	1.2	0.4–26	—/?	—
Fluoranthene	202.3	0.26	5.3–26	+/?	+ (Ames)
Pyrene	202.3	0.14	0.4 to > 90	—/?	+/? (Ames), + (USD), + (SCE)
Benzo[<i>a</i>]pyrene	252.3	0.003	0.3 to > 300	+	+ (Ames), + (USD), + (SCE), + (CA), + (DA)

+, positive; —, negative; ?, inadequate/inconclusive data; Ames, *Salmonella typhimurium* reversion assay; CA, chromosomal aberration; DA, DNA adducts; SCE, sister chromatid exchange; USD, unscheduled DNA synthesis.

^aData compiled from Cerniglia¹⁹.

^bData compiled from Wild *et al.*¹⁴⁴.

have led to the realization that a single type of sediment can contain thousands of aromatic compounds⁴⁵. PAHs have been detected in air^{85, 92}, soil and sediment^{63, 87, 88, 103, 132, 148}, surface water, groundwater, and road runoff^{14, 61, 93, 107}. They are also dispersed from the atmosphere to vegetation¹³⁶ and contaminate foods^{33, 90, 124}.

Two major sources of PAH contamination are coal and petroleum; therefore, a high level of contamination by PAHs is often found at coal- and petroleum-treatment sites, including creosote wood-treatment facilities (creosote is used as a lumber preservative and contains many PAHs); for example, the PAH contamination level has been found to be 1.79×10^6 ng/g at an oil refinery outfall in Southampton, England, while it was 5 ng/g at an undeveloped area in Alaska⁶⁸.

The possible fates of PAHs in the environment include volatilization, photo-oxidation, chemical oxidation, bioaccumulation, adsorption, and microbial degradation. Recent studies have shown the biodegradation of PAHs to be the major process resulting in the decontamination of sediment and surface soil¹²³. These compounds can be degraded to carbon dioxide and water (mineralized) or be partially transformed by microorganisms^{18, 20, 43, 125}. The bioremediation of soil or sediment contaminated with PAHs is an alternative means of detoxifying hazardous waste which may replace traditional methods such as incineration^{4, 34, 52}. In this chapter, the bacteria and metabolic pathways involved in the degradation of PAHs are summarized, and the biological constraints on PAH biodegradation are discussed.

Figure 2. Initial steps in the aerobic catabolism of a PAH molecule by bacteria.

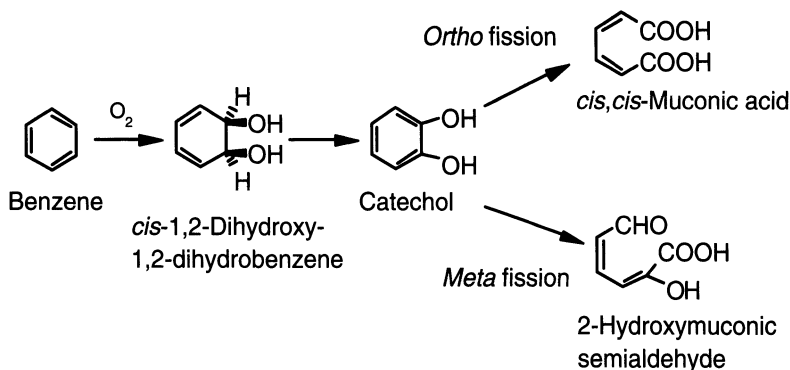


Figure 3. Ortho- and meta-ring fission pathways for the catabolism of PAHs.

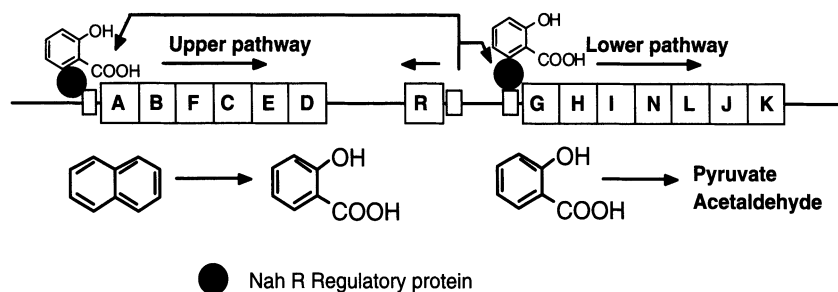


Figure 4. Organization and regulation of genes for the degradation of naphthalene in *P. putida* PpG7.

called *Pseudomonas* sp. strain U2³⁷; *phn*, *Burkholderia* sp. strain RP007⁸⁹) and chromosomes (*pah*, *P. putida* OUS82¹²⁸; *nah*, *Pseudomonas stutzeri* AN10^{11, 12}). The genes for enzymes in the naphthalene-degradation pathways in *Pseudomonas putida* PpG7 are clustered in two operons (*nah* and *sal*^{146, 147}) on plasmid NAH7. The *nah* operon encodes an upper metabolic pathway for the metabolism of naphthalene to salicylate, while the *sal* operon encodes a lower pathway for the metabolism of salicylate via catechol to pyruvate and acetaldehyde. The induction of the *nah* and *sal* operons is controlled by *nahR* which recognizes salicylate¹⁵¹ (Figure 4). The naphthalene-degradation genes on NAH7 are located on a defective transposable element¹³¹.

The genes for the initial steps in the degradation of naphthalene, phenanthrene, and dibenzothiophene have been cloned and sequenced in many strains⁵¹. The amino acid sequences of the catabolic enzymes deduced from their nucleotide sequences show high identity to each other. Less-homologous groups

of genes coding for the enzymes involved in the degradation of PAHs have been found in *Rhodococcus* sp.¹³⁰, *Comamonas testosteroni*⁴⁶ and *Nocardioides* sp.¹¹⁷. Many *Sphingomonas* strains can degrade a wide variety of PAHs; for example, *Sphingomonas* (formerly called *Pseudomonas*) *paucimobilis* EPA505 degrades naphthalene, anthracene, phenanthrene, and fluoranthene¹²⁷. The PAH-degradative genes in these *Sphingomonas* strains are distantly related to those in pseudomonads and other genera. The arrangement of the PAH-degradative genes in *Sphingomonas* is also particular, and the catabolic genes in *Sphingomonas* are encoded in a larger number of operons than that found in pseudomonads¹⁰⁶.

3. DEGRADATIVE PATHWAYS OF SELECTED PAHs

3.1. Anaerobic Degradation of Naphthalene and 2-Methylnaphthalene

The anaerobic degradation of naphthalene and 2-methylnaphthalene occurs under sulfate-reducing and nitrate-reducing conditions. 2-Naphthoic acid is the major metabolite of anaerobic naphthalene degradation and is generated by the incorporation of bicarbonate into the carboxyl group^{98, 99}. In the anaerobic pathway for the degradation of 2-methylnaphthalene, fumarate is added to the methyl group, as is the case in anaerobic toluene degradation. The product, naphthyl-2-methyl-succinic acid, is subsequently transformed to 2-naphthoic acid². Analogous to the anaerobic benzoyl-CoA pathway, 2-naphthoic acid is then reduced to hydroxydecahydro-2-naphthoic acid and subsequently oxidized to oxodecahydro-2-naphthoic acid³ (Figure 4). The marine sulfate-reducing bacterium, strain NaphS2, that grew with naphthalene under sulfate-reducing conditions has been isolated, this strain being affiliated with sulfate-reducing bacteria in the δ -subclass of the Proteobacteria³⁹. More recently, three pure bacterial cultures which degraded naphthalene under nitrate-reducing conditions have been established. These were designated NAP-3-1, NAP-3-2, and NAP-4. Phylogenetic analyses based on 16S rRNA gene sequences have shown that NAP-3-1 was closely related to *P. stutzeri*, while NAP-4 was closely related to *Vibrio pelagius*¹¹¹.

3.2. Aerobic Degradation of Two- and Three-Ring PAHs (Naphthalene, Phenanthrene, and Anthracene)

The bacterial degradation of naphthalene, one of the simplest PAHs, has been reported more extensively than that of any other PAHs, and most of the

studies on aerobic naphthalene biodegradation have been conducted with microorganisms belonging to the genus *Pseudomonas*^{20, 43, 126, 142}. The initial step involves incorporating both atoms of molecular oxygen into the substrate to form *cis*-1,2-dihydroxy-1,2-dihydronaphthalene, which is further metabolized via 1,2-dihydroxynaphthalene, 2-hydroxychromene-2-carboxylic acid (HCCA), *trans*-*o*-hydroxybenzylidenepyruvic acid (*o*HBPA), salicylaldehyde, salicylic acid, and either catechol or gentisic acid^{32, 150} (Figure 5).

The bacterial degradation of biphenyl, another simple PAH, and its chlorinated derivatives are reviewed in the next chapter of this volume (Chapter 18, Volume 3).

Phenanthrene, a three-ring angular PAH, is known to be a human skin photosensitizer and mild allergen and is mutagenic to bacterial systems under certain conditions³⁶. It has also been found to be an inducer of sister chromatid exchange and a potent inhibitor of gap-junctional intercellular communication¹³⁸.

The pathways for phenanthrene metabolism by bacteria have been reported by several investigators^{1, 8, 41, 43, 64–66, 81, 82, 117}. In general, the initial reaction is the action of a dioxygenase with subsequent oxidation to form 3,4-dihydroxyphenanthrene, which then undergoes *meta*-cleavage and is converted to 1-hydroxy-2-naphthoic acid. This is the common upper route of the phenanthrene degradation pathways so far identified. 1-hydroxy-2-naphthoic acid can be further degraded by three different pathways (Figure 6). *Pseudomonas* strains convert it to 1,2-dihydroxynaphthalene, which is then subject to *meta*-cleavage to form salicylic acid⁴³. A series of reactions for the conversion of phenanthrene to 1-hydroxynaphthoic acid (the phenanthrene pathway) is mechanistically identical to that for the conversion of naphthalene to salicylic acid (the naphthalene pathway), and each step of the reactions is catalyzed by an enzyme common to the phenanthrene and naphthalene pathways in *P. putida* OUS82⁸⁵. Similarly, the conversion of 1-hydroxy-2-naphthoic acid to 1,2-dihydroxynaphthalene seems to be catalyzed by a broad-specificity salicylate hydroxylase (salicylate-1-monooxygenase) in *P. putida* BS202-P1. In other words, the enzymes of the naphthalene-degradative pathway are sufficient to catalyze the mineralization of phenanthrene if these enzymes exhibit relaxed substrate specificity⁵. Salicylic acid can be further degraded via the formation of either catechol or gentisic acid. Both catechol and gentisic acid undergo ring fission to form the TCA cycle intermediates⁶². *Pseudomonas* sp. DLC-P11 converts 1-hydroxy-2-naphthoic acid to 1-naphthol, which is degraded via 4-hydroxy-1-tetralone to form *o*-phthalic acid¹¹⁸. *Aeromonas*, *Vibrio*, *Alcaligenes*, and *Nocardioides* convert 1-hydroxy-2-naphthoic acid to *o*-phthalic acid and protocatechuic acid which is finally cleaved to form pyruvic acid and ultimately enters the TCA cycle^{41, 62, 64–66, 83}.

The genes for the phenanthrene metabolism pathway via salicylic acid and catechol have been isolated from several bacterial strains. The *pah* genes cloned from *P. putida* strain OUS82 show a high degree of homology to the

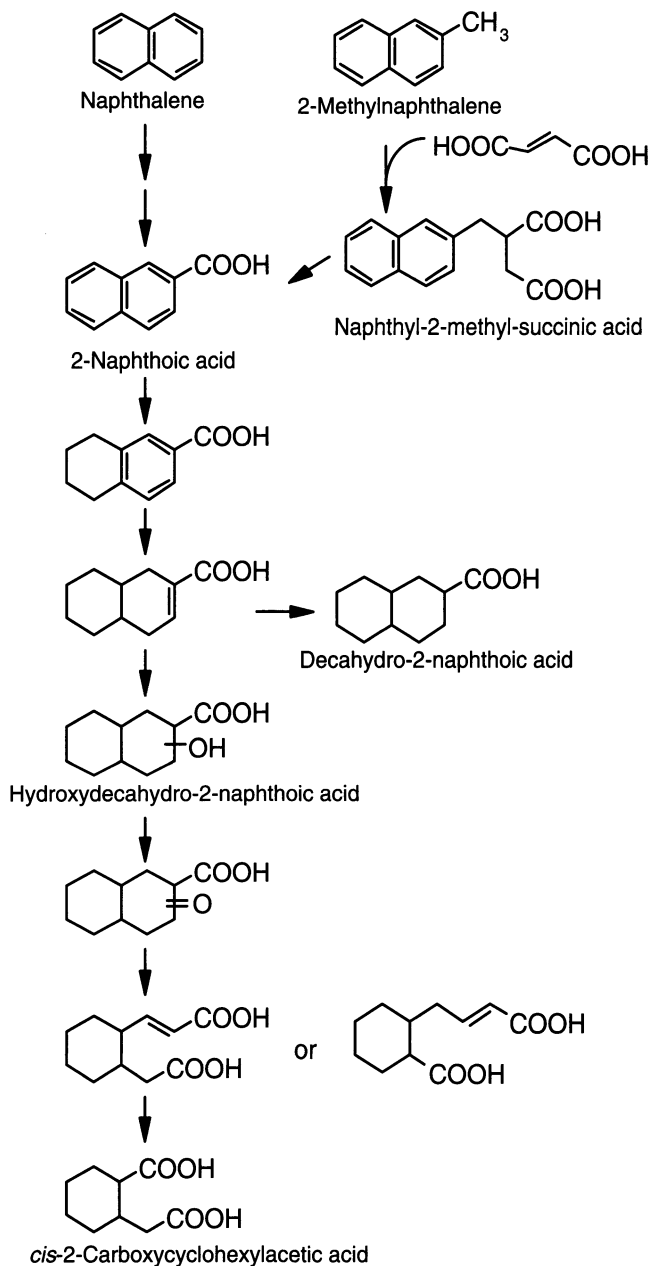


Figure 5. Proposed pathway for the anaerobic degradation of naphthalene and 2-methylnaphthalene^{2,3}.

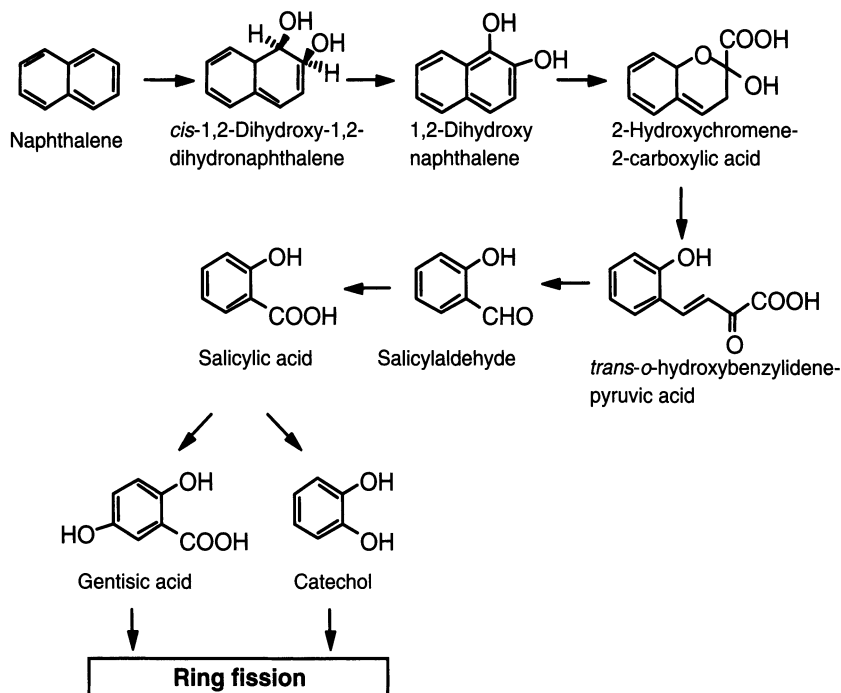


Figure 6. Pathway for the bacterial metabolism of naphthalene.

nah genes^{83, 145}. The amino acid sequences of these enzymes^{128, 145} are more than 90% identical. This observation has led to the expectation that the sequence diversity in the genes for the degradation of naphthalene and phenanthrene in bacteria would be limited. However, this view has recently been modified. The *phn* genes from *Burkholderia* sp. strain RP007 have been shown to be significantly different in sequence and gene order from the previously characterized naphthalene- or phenanthrene-degrading genes⁸⁹.

The genes for the protocatechuate pathway have been isolated from *Nocardioides* sp. strain KP7 which is capable of growing on phenanthrene but not on naphthalene^{116, 117}. The *phd* genes are separated into two clusters: one is the *phdEFABGHCD* gene cluster encoding the enzymes responsible for the transformation of phenanthrene to 1-hydroxy-2-naphthoate, and the other is the *phdIJK* gene cluster encoding the enzymes for the transformation of 1-hydroxy-2-naphthoate to *o*-phthalate¹¹⁶.

The growth rates of bacteria on naphthalene, phenanthrene, and anthracene appear to be related to the solubility in water of these PAHs¹³⁵. Since the solubility of anthracene in water is only 0.07 mg/l³¹ (Table 1),

reports of organisms that can grow with anthracene as the sole source of carbon and energy are relatively rare. The bacterial mineralization of anthracene is achieved by the pathway shown in Figure 7. This degradation pathway is quite similar to that described for naphthalene. For example, *Pseudomonas aeruginosa* metabolizes anthracene via anthracene *cis*-1,2-dihydrodiol, 1,2-dihydroxyanthracene, *cis*-4-(2-hydroxynaphth-3-yl)-2-oxo-but-3-enoic acid, 2-hydroxy-3-naphthaldehyde, 2-hydroxy-3-naphthoic acid, and 2,3-dihydroxynaphthalene³⁵. The latter compound is mineralized via salicylic acid and catechol by the enzymes of the naphthalene pathway¹⁴².

Alternative pathways for the degradation of anthracene and phenanthrene exist in Gram-positive bacteria. In anthracene-grown cultures of *Rhodococcus* sp. and *Mycobacterium vanbaalenii* strain PYR-1, two ring-cleavage products of 1,2-dihydroxyanthracene, namely, 6,7-benzocoumarin formed by *meta*-cleavage and 3-(2-carboxyvinyl)naphthalene-2-carboxylic acid formed by *ortho*-cleavage, have been detected^{26, 100}. Furthermore, 1-methoxy-2-hydroxyanthracene and 9,10-anthraquinone have been found in *M. vanbaalenii* strain PYR-1. The pathways for the degradation of anthracene in Gram-positive bacteria have been proposed from the results¹⁰⁰ (Figure 7).

3.3. Aerobic Degradation of PAHs Composed of more than Three Rings

Although the catabolism of PAHs possessing three or fewer fused aromatic rings has been well characterized, little is known about the bacterial catabolism of high-molecular-weight PAHs such as benz[*a*]anthracene, chrysene, and benzo[*a*]pyrene. Research pertaining specifically to the bacterial biodegradation of PAHs composed of more than three rings have been previously summarized⁷¹.

Of the four-ring PAHs, the biodegradation of fluoranthene, pyrene, chrysene, and benzo[*a*]anthracene have been investigated to various degrees. Fluoranthene is a PAH containing a five-membered ring. The utilization of fluoranthene as the sole source of carbon and energy by a pure bacterial strain was first described in 1990 by two independent research groups^{101, 139}. After this, several other fluoranthene-utilizing strains have been isolated^{9, 23, 60, 69, 84, 109, 120} and at least three different pathways have been proposed (Figure 8). For *Achromobacter xylosoxidans* subsp. *denitrificans* (formerly called *Alcaligenes denitrificans*) strain WW1, Weissenfels *et al.*¹³⁹ have proposed an initial attack at the 7,8-position of the benzylic moiety of the fluoranthene skeleton (Figure 8, left column) which, after ring opening between positions 6b and 7, yields two metabolic intermediates: 1-acenaphthenone and subsequently 3-hydroxymethylbenzo[*d,e*]chromen-2-one. Naphthalene-1,8-dicarboxylic acid as a fluoranthene

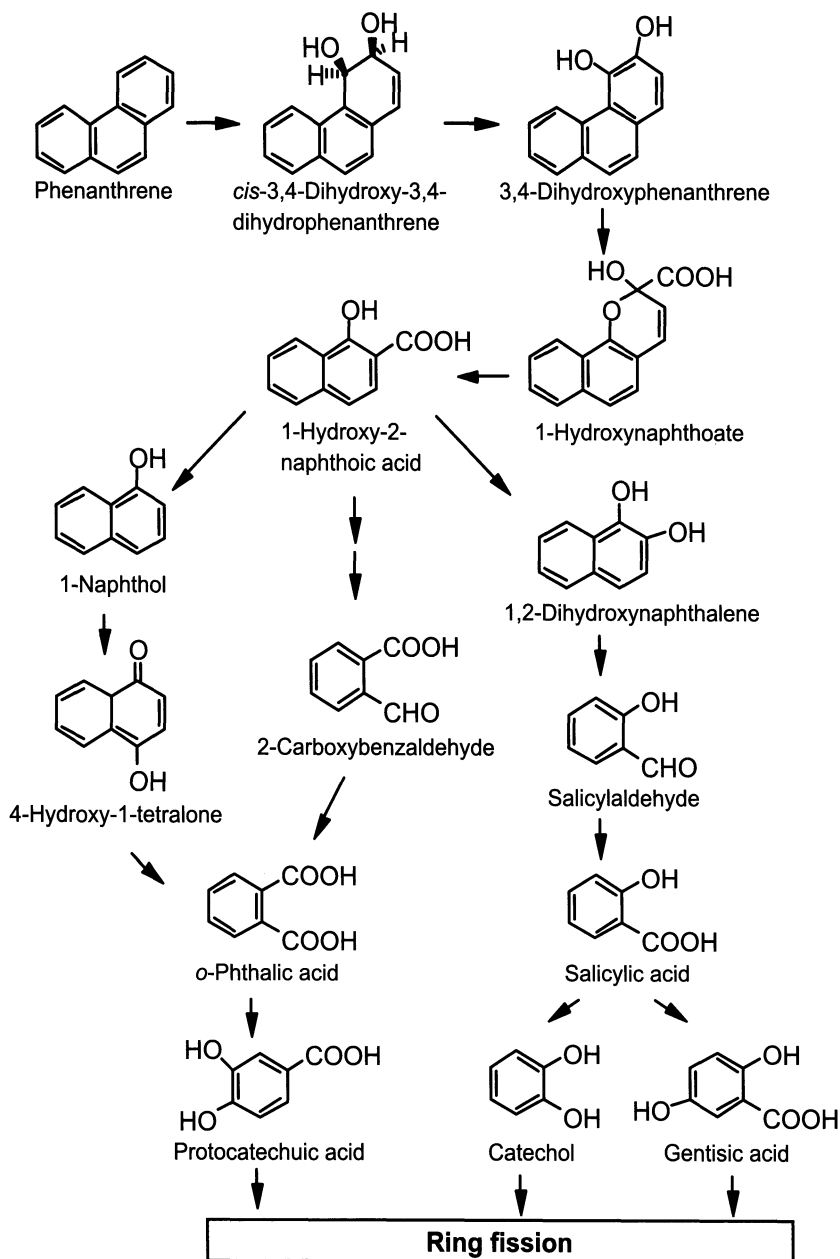


Figure 7. Pathway for the bacterial metabolism of phenanthrene.

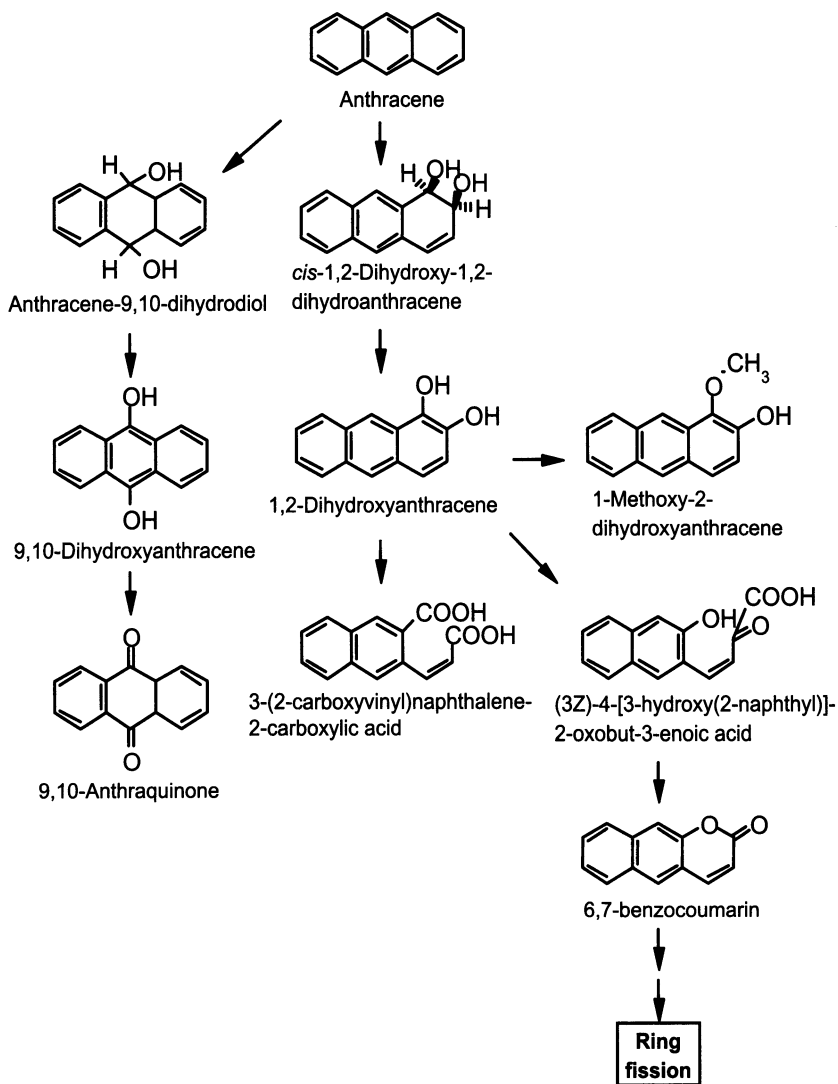


Figure 8. Pathway for the bacterial metabolism of anthracene by *P. aeruginosa*.

metabolite of *S. paucimobilis* EPA505 has been described by Mueller *et al.*¹⁰¹. *Mycobacterium* sp. PYR1 metabolizes fluoranthene via *cis*-1,2-fluoranthene dihydrodiol, 1-carboxylic-9-hydroxyfluorene, 1-carboxy-fluorene-9-one, 9-hydroxyfluorene, and fluorene-9-one (Figure 8, middle column). The late fluoranthene degradation products observed with these bacteria include 2-carboxybenzaldehyde, phthalic acid, phenylacetic acid, benzoic acid, and

adipic acid⁷⁶⁻⁷⁸. *Mycobacterium* sp. strain KR20 metabolizes fluoranthene via *cis*-2,3-fluoranthene dihydrodiol, *Z*-9-carboxymethylene-fluorene-1-carboxylic acid, *cis*-1,9a-dihydroxy-1-hydro-fluorene-9-one-8-carboxylic acid, 4-hydroxybenzochromene-6-one-7-carboxylic acid, and benzene-1,2,3-tricarboxylic acid (Figure 9, right column).

The bacterial degradation of pyrenes, a pericondensed type of PAH, has been reported by a number of groups, and some have identified the metabolites and proposed pathways^{21, 49, 56, 134}. Seven metabolites of pyrene metabolism, *cis*-4,5-pyrenedi hydrodiol, *trans*-4,5-pyrenedi hydrodiol, pyrenol, 4-hydroxy-perinaphthenone, 4-phenanthroic acid, phthalic acid, and cinnamic acid, were detected for *M. vanbaalenii* strain PYR-1. *M. vanbaalenii* strain AP1 initiates its attack on pyrene by either monooxygenation or dioxygenation at its C-4 and C-5 positions to respectively give *trans*- and *cis*-4,5-dihydroxy-4,5-dihydropyrene¹³⁴. Dehydrogenation of the latter, *ortho* cleavage of the resulting diol to form phenanthrene 4,5-dicarboxylic acid, and subsequent decarboxylation to phenanthrene 4-carboxylic acid lead to the degradation of phenanthrene 4-carboxylic acid via phthalate¹⁹ (Figure 9). In contrast, *Rhodococcus* sp. produces a different set of metabolites from pyrene, including 1,2- and 4,5-dihydroxypyrene, *cis*-2-hydroxy-3-(perinaphthenone-9-yl) propionic acid, and 2-hydroxy-2-(phenanthrene-5-one-4-enyl)acetic acid¹³⁷. The genes involved in the initial degradation of PAHs in *M. vanbaalenii* strain PYR-1⁸⁰ have recently been reported (Figure 10).

Although many of the high-molecular-weight PAH-degrading bacteria described are actinomycetes, Gram-negative bacteria, for example, *Stenotrophomonas maltophilia* strain VUN 10,003 (formerly classified as *Burkholderia cepacia*; refs [68], [69]), *Stenotrophomonas paucimobilis* EPA 505¹⁰⁸ and *Pseudomonas fluorescens*¹⁷, can also degrade a variety of high-molecular-weight PAHs. A variety of non-actinomycete bacteria have also been reported to metabolize fluoranthene, pyrene, chrysene, and benz[*a*]anthracene.

There is currently only limited information regarding the bacterial biodegradation of PAHs with five or more rings in both environmental samples and pure or mixed cultures. Most studies have been focused on the five-ring benzo[*a*]pyrene molecule due its potential hazards to human health. Benzo[*a*]pyrene biodegradation by pure and mixed cultures of bacteria has been shown to occur, although bacteria capable of utilizing benzo[*a*]pyrene as the sole source of carbon and energy have never been demonstrated. All reported benzo[*a*]pyrene biotransformations by bacteria have therefore occurred under co-metabolic conditions. Early observations of benzo[*a*]pyrene biodegradation were made with mutant *Sphingomonas yanoikuyae* (formerly called *Beijerinckia* sp.) strain B8/36 grown on succinate plus biphenyl⁴⁴ and with *Pseudomonas* strain NCBI 9816 grown on succinate plus salicylate⁷.

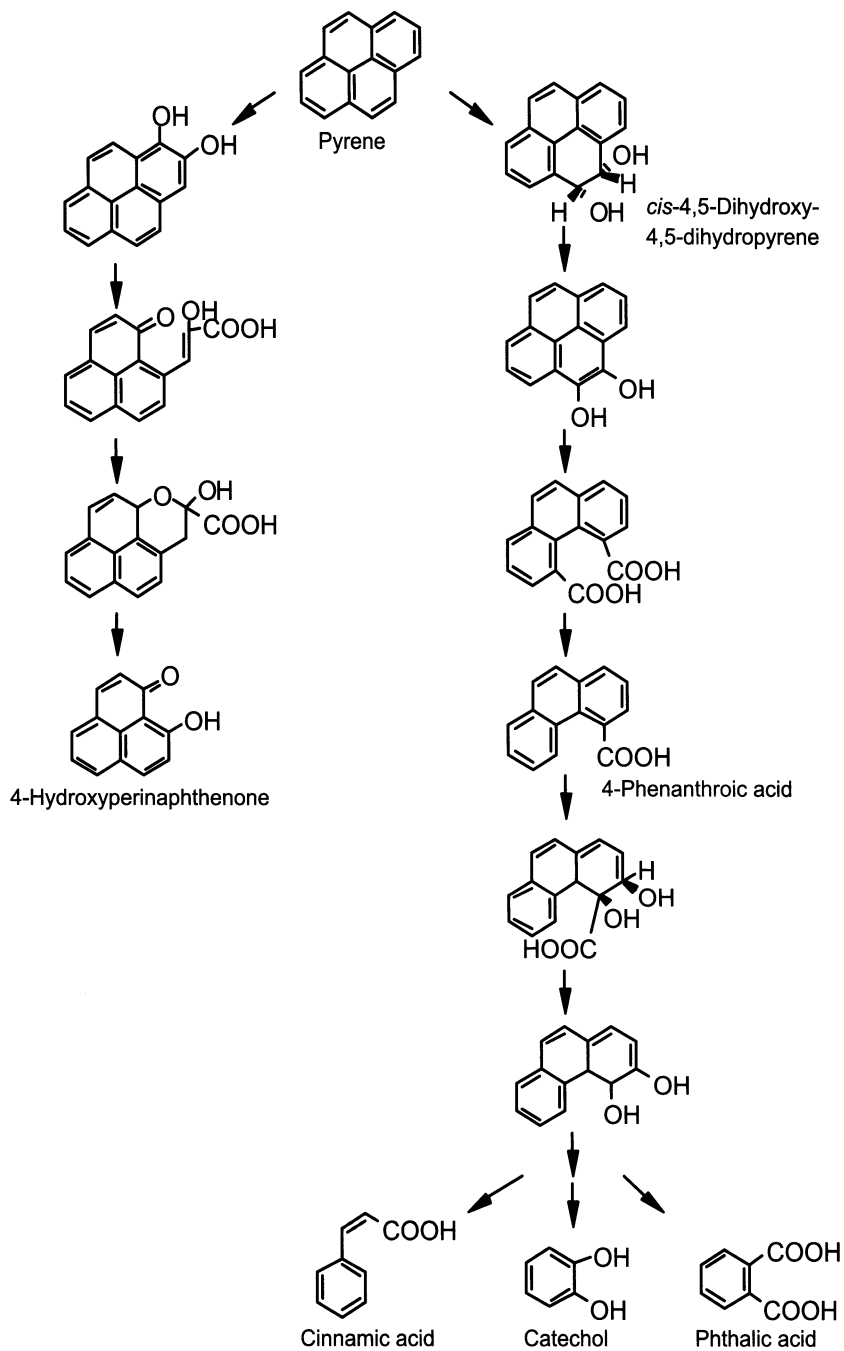


Figure 10. Metabolism of pyrene by *Mycobacterium* strains.

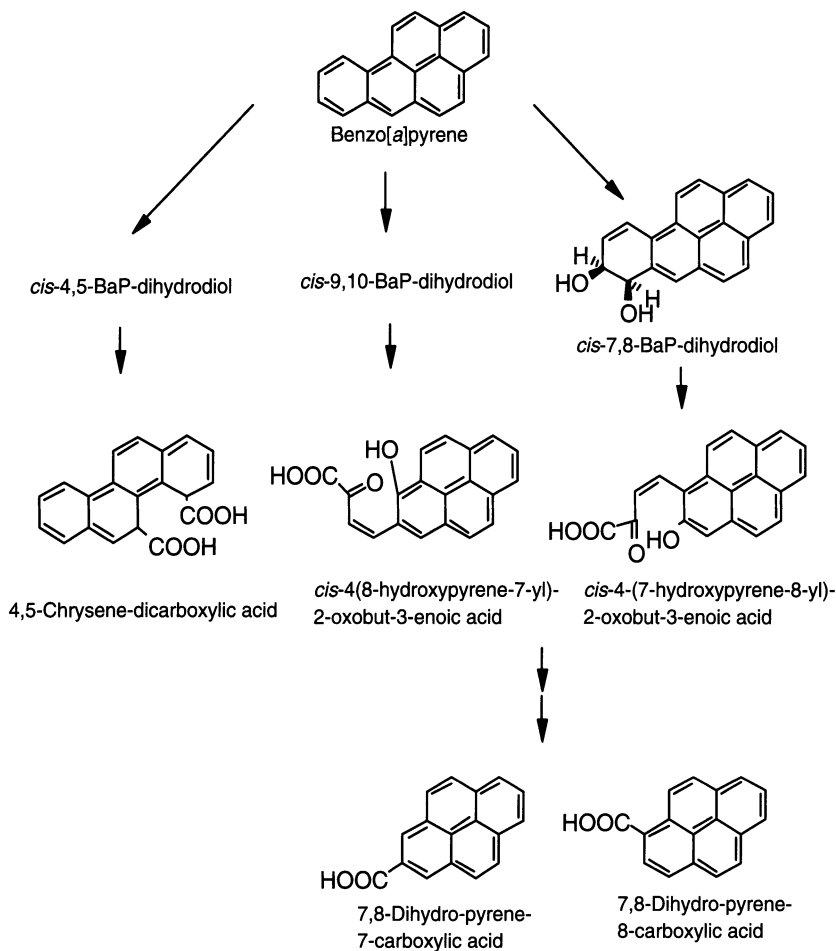


Figure 11. Proposed pathways for the metabolism of benzo[a]pyrene by *Mycobacterium* sp. strain RJGII-135¹¹⁹. The structures of the identified metabolites are shown.

Figure 11, the pathways for the metabolism of benzo[a]pyrene have been proposed.

4. BIOAVAILABILITY OF PAHs

It has been found in field tests on the bioremediation of contaminated soil with white rot fungi that PAHs with four or more fused rings were not degraded. This result was interpreted as indicating that these PAHs were adsorbed to the soil, and were not accessible to biodegradation²⁵. Soil is

a mixture of mineral and organic materials, and is an effective adsorbent for PAHs. The adsorption capacity is influenced by the nature of the soil, the soil moisture, and other factors. The PAH-binding sites on soil could be classified into two groups: those on the mineral surface and those on organic matter. If the soil is wet, most of the mineral surface is occupied by water, and PAHs would be mainly adsorbed to the organic matter. In dry soil, however, the adsorption of PAHs to both the mineral surface and organic matter would occur. It has been demonstrated that a significant portion of adsorbed anthracene and other PAHs became non-extractable, especially when the water content of the soil was low. When the soil was dry, adsorbed PAHs were polymerized and became non-available to biodegradation. The involvement of transient metals on the mineral surface has been suggested for the polymerization of PAHs⁷³.

Low solubility in water and adsorption to soil and sediment are two major factors of PAHs that limit their availability to microorganisms, and the enhancement of bioavailability seems to require bacteria to utilize high-molecular-weight PAHs. High-affinity uptake systems may be one of such bioavailability-enhancing mechanisms. The uptake of naphthalene has been investigated with *Pseudomonas fluorescens*. The uptake was significantly inhibited by azide or 2,4-dinitrophenol, suggesting that an energy-linked transport system was involved in the naphthalene uptake¹⁴⁰. A later study using several inhibitors of respiration and membrane transport (azide, cyanide, and carbonyl cyanide *m*-chlorophenyl hydrazone), however, provided the opposite conclusion: the results suggested that the incorporation of PAHs inside the cells was mediated by passive diffusion, while an energy-driven efflux system to transport PAHs out of the cell exists¹⁵. The presence of an active efflux pump in *P. fluorescens* appears to prevent the efficient metabolism of PAHs. However, it has been experimentally demonstrated that the efflux system did not affect the metabolism of PAHs¹⁵. The affinity of the PAH-degradative enzyme is probably much higher than that of the efflux system.

The outer membrane of Gram-negative bacteria can be a barrier to the PAH uptake if the outer surface of the membrane has a hydrophilic nature. Although the outer-membrane pore for the passage of monoaromatic hydrocarbons has been demonstrated in *P. putida* harboring TOL plasmid pWW0⁷⁴, an outer-membrane pore for the passage of PAHs is not known.

PAHs which have limited solubility in water tend to be sorbed to hydrophobic materials or to be dissolved in organic solvents. The adhesion of cells to a PAH-sorbing hydrophobic surface or the attachment of cells to the interface between aqueous and PAH-dissolving organic phases would reduce the distance between the cells and the PAH substrate. PAHs then would be more readily accessible to the adhering cells. *Mycobacterium* sp. LB501T grows on solid anthracene as a confluent biofilm. The adhesion of the

bacterium to the surface of solid anthracene is induced by this growth on anthracene. The cell surface of anthracene-grown cells is more hydrophobic than that of glucose-grown cells. It has been suggested that the attachment and biofilm formation may be a specific response of *Mycobacterium* sp. LB501T to optimize the substrate bioavailability¹⁴¹. The kinetics of phenanthrene utilization in *P. aeruginosa* strain 19ST prompted the suggestion that cells associated at the interface between the aqueous and non-aqueous phases had selective advantages and grew more rapidly than cells harbored in the aqueous phase⁴⁰.

Cell motility in combination with chemotactic activity toward a PAH renders the PAH substrate more accessible to bacterial cells. Chemotaxis to naphthalene has been demonstrated in *P. putida* strain G7, and the gene responsible for chemotaxis, *nahY*, has been identified. This gene is cotranscribed with the *meta* cleavage pathway genes on the NAH7 catabolic plasmid for naphthalene degradation. The presence of *nahY* on the catabolic plasmid implies that chemotaxis is important for the degradation of naphthalene in a natural environment^{48, 105}.

Surfactants exhibit several particular characteristics: first, they form aggregates called micelles above the critical micelle concentration (CMC); second, they are accumulated at the water surface or at the interface between two immiscible liquids because of their amphipathic nature, and thereby reduce the surface or interfacial tension; third, they enhance the formation of an emulsion of two immiscible liquids. As mentioned earlier, the low solubility in water and the adsorption to hydrophobic substances are two major factors of PAHs that limit their availability to microorganisms; however, the availability can be expected to increase when PAHs are dissolved by a surfactant. To support this expectation, the rate of pyrene degradation by *S. maltophilia* strain VUN 10,010 was enhanced by the addition of some but not all nonionic surfactants^{10, 70}. The mineralization of benzo[a]pyrene by a microbial consortium was stimulated by a water-soluble compound produced by *Rhodanobacter* sp. strain BPC1. This compound seemed to solubilize benzo[a]pyrene⁷². Thus, the production of surface-active compounds by microorganisms is considered to be an important microbial process that increases the bioavailability of high-molecular-weight PAHs.

However, the situation is not that simple. Conflicting results concerning the effect of surfactants on PAH biodegradation have been obtained: surfactants stimulated biodegradation in some cases, while they were inhibitory in others^{16, 67, 113, 115, 129}. Several hypotheses have been proposed to explain the inhibitory effect of a surfactant on biodegradation¹⁴³. One theory is that the speed of PAH biodegradation is retarded by a surfactant if microorganisms cannot utilize PAH entrapped within the micelles of the surfactant. However, studies on the micelle-exchange dynamics of hydrophobic compounds in the

aqueous phase have indicated that both the entry and exit rates of PAHs were likely to be far higher than the biodegradation rate⁴⁷. Other hypotheses have proposed either that biodegradation at a high surfactant concentration would be inhibited if the surfactant or dissolved PAH were toxic, or that the surfactant would be preferentially used by microorganisms as the source of carbon and energy. The real situation would be more complex than that indicated by any of these hypotheses, because some observations concerning the effects of surfactants remain unexplained; for example, Brij 30 inhibited pyrene mineralization by a *P. putida* strain in an aqueous culture, while the same surfactant promoted it when soil was added to the aqueous culture²⁹. The reason for this dual effect of the surfactant is not clear.

6. CONCLUSIONS

Although knowledge regarding the bacterial biodegradation of PAHs has been advanced in the last decade, we do not yet know many aspects of PAH biodegradation. Further study is required to understand the organization of PAH-degradative genes and the regulatory mechanism for the expression of these genes. More effort needs to be directed to characterize the enzymes and intermediates of the PAH-degradative pathways. Our understanding of the bioavailability of PAHs is also preliminary. The relationship between cell surface properties and the efflux of PAHs through the cell membranes should also be established. In addition, elucidation of the active transport mechanisms for the entry of PAHs into and exit from bacterial cells is necessary to evaluate the speeds of the mass transfer and subsequent biodegradation of PAHs inside the bacterial cells. The effects of biosurfactants and synthetic surfactants on the biodegradation of PAHs are complex issues that also require more investigation. The structures of biosurfactants are diverse²⁸ and the physiological roles of biosurfactants may also be diverse^{102, 112}. To understand the effect of a biosurfactant or a synthetic surfactant on PAH biodegradation, it is necessary to identify the relevant parameters so that a model can be built for each surfactant, each PAH species, and each PAH-degrading bacterium.

The degradation of PAHs in a natural environment is often slow due to such factors as nutrients, bioavailability of PAHs (sorption to particles), oxygen, and presence of PAH-degrading microorganisms. Subsurface organic-impacted sediment is commonly anaerobic. Although the aerobic bacterial degradation of PAHs has been well documented, PAHs had been thought to be recalcitrant to biodegradation without oxygen. However, recent studies have demonstrated PAH biodegradation under anaerobic conditions, although little is known about the bacteria responsible for the anaerobic activities to

degrade PAHs. Molecular techniques for environmental microbiology will be an aid to the detection and isolation of anaerobic PAH-degrading bacteria from environmental samples. Further knowledge of the biochemical reactions, enzymes, and genetics of anaerobic PAH biodegradation will also be useful to design bioremediation methods for enhancing the removal of PAHs under anaerobic conditions.

REFERENCES

1. Adachi, K., Iwabuchi, T., Sano, H., and Harayama, S., 1999, Structure of the ring cleavage product of 1-hydroxy-2-naphthoate, an intermediate of the phenanthrene-degradative pathway of *Nocardioides* sp. strain KP7. *J. Bacteriol.*, 181:757–763.
2. Annweiler, E., Materna, A., Safinowski, M., Kappler, A., Richnow, H.H., Michaelis, W., and Meckenstock, R.U., 2000, Anaerobic degradation of 2-methylnaphthalene by a sulfate-reducing enrichment culture. *Appl. Environ. Microbiol.*, 66:5329–5333.
3. Annweiler, E., Michaelis, W., and Meckenstock, R.U., 2002, Identical ring cleavage products during anaerobic degradation of naphthalene, 2-methylnaphthalene, and tetralin indicate a new metabolic pathway. *Appl. Environ. Microbiol.*, 68:852–858.
4. Aronstein, B.N., Cavillo, Y.M., and Alexander, M., 1991, Effect of surfactants at low concentrations on the desorption and biodegradation of sorbed aromatic compounds in soil. *Environ. Sci. Technol.*, 25:1728–1731.
5. Balashova, N.V., Stolz, A., Knackmuss, H.J., Kosheleva, I.A., Naumov, A.V., and Boronin, A.M., 2001, Purification and characterization of a salicylate hydroxylase involved in 1-hydroxy-2-naphthoic acid hydroxylation from the naphthalene and phenanthrene-degrading bacterial strain *Pseudomonas putida* BS202-P1. *Biodegradation*, 12:179–188.
6. Banerjee, D.K., Fedorak, P.M., Hashimoto, A., Masliyah, J.H., Pickard, M.A., and Gray, M.R., 1995, Monitoring the biological treatment of anthracene-contaminated soil in a rotating-drum bioreactor. *Appl. Microbiol. Biotechnol.*, 43:521–528.
7. Barnsley, E.A., 1975, The bacterial degradation of fluoranthene and benzo[a]pyrene. *Can. J. Microbiol.*, 21:1004–1008.
8. Barnsley, E.A., 1983, Phthalate pathway of phenanthrene metabolism formation of 2'-carboxybenzalpyruvate. *J. Bacteriol.*, 154:113–117.
9. Boldrin, B., Thiem, A., and Fritsche, C., 1993, Degradation of phenanthrene, fluorene, fluoranthene, and pyrene by a *Mycobacterium* sp. *Appl. Environ. Microbiol.*, 59:1927–1930.
10. Boonchan, S., Britz, M.L., and Stanley, G.A., 1998, Surfactant-enhanced biodegradation of high molecular weight polycyclic aromatic hydrocarbons by *Stenotrophomonas maltophilia*. *Biotechnol. Bioeng.*, 59:482–494.
11. Bosch, R., Garcia-Valdes, E., and Moore, E.R.B., 1999, Genetic characterization and evolutionary implications of a chromosomally encoded naphthalene-degradation upper pathway from *Pseudomonas stutzeri* AN10. *Gene*, 236:149–157.
12. Bosch, R., Garcia-Valdes, E., and Moore, E.R., 2000, Complete nucleotide sequence and evolutionary significance of a chromosomally encoded naphthalene-degradation lower pathway from *Pseudomonas stutzeri* AN10. *Gene*, 245:65–74.
13. Bossert, I.D. and Bartha, R., 1986, Structure biodegradability relationships of polycyclic aromatic hydrocarbons in soil. *Bull. Environ. Contam. Toxicol.*, 37:490–495.
14. Boxall, A.B.A. and Maltby, L., 1997, The effects of motorway runoff on freshwater ecosystems. 3. Toxicant confirmation. *Arch. Environ. Contam. Toxicol.*, 33:9–16.

15. Bugg, T., Foght, J.M., Pickard, M.A., and Gray, M.R., 2000, Uptake and active efflux of polycyclic aromatic hydrocarbons by *Pseudomonas fluorescens* LP6a. *Appl. Environ. Microbiol.*, 66:5387–5392.
16. Bury, S.J. and Miller, C.A., 1993, Effect of micellar solubilization on biodegradation rates of hydrocarbons. *Environ. Sci. Technol.*, 27:104–110.
17. Caldini, G., Cenci, G., Manenti, R., and Morozzi, G., 1995, The ability of an environmental isolate of *Pseudomonas fluorescens* to utilize chrysene and other four-ring polynuclear aromatic hydrocarbons. *Appl. Microbiol. Biotechnol.*, 44:225–229.
18. Cerniglia, C.E., 1984, Microbial metabolism of polycyclic aromatic hydrocarbons. *Adv. Appl. Microbiol.*, 30:31–37.
19. Cerniglia, C.E., 1992, Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation*, 3:351–368.
20. Cerniglia, C.E. and Heitkamp, M.A., 1989, Microbial degradation of polycyclic aromatic hydrocarbons (PAHs) in aquatic environment. In U. Varanasi (ed.), *Metabolism of Polycyclic Aromatic Hydrocarbons in the Aquatic Environment*, pp. 41–68. CRC Press, Inc., Boca Raton, FL.
21. Cerniglia, C.E. and Heitkamp, M.A., 1990, Polycyclic aromatic hydrocarbon degradation by *Mycobacterium*. *Methods Enzymol.*, 188:148–153.
22. Coates, J.D., Woodward, J., Allen, J., Philip, P., and Lovley, D.R., 1997, Anaerobic degradation of polycyclic aromatic hydrocarbons and alkanes in petroleum-contaminated marine harbor sediments. *Appl. Environ. Microbiol.*, 63:3589–3593.
23. Dagher, F., Deziel, E., Lirette, P., Paquette, G., Bisailon, J.-G., and Villemur, R., 1997, Comparative study of five polycyclic aromatic hydrocarbon degrading bacterial strains isolated from contaminated soils. *Can. J. Microbiol.*, 46:368–377.
24. Dagley, S., 1978, Pathways for the utilization of organic growth substrates. *The Bacteria*, 6:305–388.
25. Davis, M.W., Glaser, J.A., Evans, J.W., and Lamar, R.T., 1933, Field evaluation of the lignin-degrading fungus *Panerochaete sordida* to treat creosote-contaminated soil. *Environ. Sci. Tech.*, 27:2572–2576.
26. Dean-Ross, D., Moody, J.D., Freeman, J.P., Doerge, D.R., and Cerniglia, C.E., 2001, Metabolism of anthracene by a *Rhodococcus* species. *FEMS Microbiol. Lett.*, 204:205–211.
27. Denome, S.A., Stanley, D.C., Olson, E.S., and Young, K.D., 1993, Metabolism of dibenzothiophene and naphthalene in *Pseudomonas* strains: Complete DNA sequence of an upper naphthalene catabolic pathway. *J. Bacteriol.*, 175:6890–6901.
28. Desai, J.D. and Banat, I.M., 1997, Microbial production of surfactants and their commercial potential. *Microbiol. Mol. Biol. Rev.*, 61:47–64.
29. Doong, R.A. and Lei, W.G., 2003, Solubilization and mineralization of polycyclic aromatic hydrocarbons by *Pseudomonas putida* in the presence of surfactant. *J. Hazard. Mater.*, 96:15–27.
30. Dunn, N.W. and Gunsalus, I.C., 1973, Transmissible plasmid coding for the early enzymes of naphthalene oxidation in *Pseudomonas putida*. *J. Bacteriol.*, 114:974–979.
31. Eastcott, L., Shiu, W.T., and Mackay, D., 1988, Environmentally relevant physical-chemical properties of hydrocarbons: A review of data and development of simple correlations. *Oil Chem. Pollut.*, 4:191–216.
32. Eaton, R.W. and Chapman, P.J., 1992, Bacterial metabolism of naphthalene: Construction and use of recombinant bacteria to study ring cleavage of 1,2-dihydroxynaphthalene and subsequent reactions. *J. Bacteriol.*, 174:7542–7554.
33. Edwards, N.T., 1983, Polycyclic aromatic hydrocarbons (PAHs) in the terrestrial environment—a review. *J. Environ. Qual.*, 12:427–441.
34. Ellis, B., Harold, P., and Kronberg, H., 1991, Bioremediation of a creosote contaminated site. *Environ. Technol.*, 12:447–459.

35. Evans, W.C., Fernley, H.N., and Griffiths, E., 1965, Oxidative metabolism of phenanthrene and anthracene by soil *Pseudomonads*; the ring fission mechanism. *Biochem. J.*, 95: 819–821.
36. Fawell, J.K. and Hunt, S., 1988, The polycyclic aromatic hydrocarbons. In J.K. Fawell and S. Hunt (eds), *Environmental Toxicology: Organic Pollutants*, pp. 241–269. Ellis Horwood, West Sussex.
37. Fuenmayor, S.L., Wild, M., Boyes, A.L., and Williams, P.A., 1998, A gene cluster encoding steps in conversion of naphthalene to gentisate in *Pseudomonas* sp. strain U2. *J. Bacteriol.*, 180:2522–2530.
38. Fujikawa, K., Fort, F.L., Samefima, K., and Sakamoto, Y., 1993, Genotoxic potency in *Drosophila melanogaster* of selected aromatic amines and polycyclic aromatic hydrocarbons as assayed in the DNA repair test. *Mutat. Res.*, 290:175–182.
39. Galushko, A., Minz, D., Schink, B., and Widdel, F., 1999, Anaerobic degradation of naphthalene by a pure culture of a novel type of marine sulphate-reducing bacterium. *Environ. Microbiol.*, 1:415–420.
40. García-Juncó, M., De Olmedo, E., and Ortega-Calvo, J.J., 2001, Bioavailability of solid and non-aqueous phase liquid (NAPL)-dissolved phenanthrene to the biosurfactant-producing bacterium *Pseudomonas aeruginosa* 19SJ. *Env. Microbiol.*, 3:561–569.
41. Ghosh, D.K. and Mishra, A.K., 1983, Oxidation of phenanthrene by strain of *Micrococcus*: Evidence of protocatechuate pathway. *Curr. Microbiol.*, 9:219–224.
42. Gibson, D.T. and Parales, R.E., 2000, Aromatic hydrocarbon dioxygenases in environmental biotechnology. *Curr. Opin. Biotechnol.*, 11:236–243.
43. Gibson, D.T. and Subramanian, V., 1984, Microbial degradation of aromatic hydrocarbons. In D.T. Gibson (ed.), *Microbial Degradation of Organic Compounds*, pp. 181–252. Dekker, New York.
44. Gibson, D.T., Venkatanayarana, M., Jerina, D.M., Yagi, H., and Yeh, H., 1975, Oxidation of the carcinogens benzo[a]pyrene and benzo[a]anthracene to dihydrodiols by a bacterium. *Science*, 189:295–297.
45. Giger, W. and Blumer, M., 1974, Polycyclic aromatic hydrocarbons in the environment; isolation and characterization by chromatography, visible, ultraviolet and mass spectrometry. *Anal. Chem.*, 46:1663–1671.
46. Goyal, A.K. and Zylstra, G.J., 1996, Molecular cloning of novel genes for polycyclic aromatic hydrocarbon degradation from *Comamonas testosteroni* GZ39. *Appl. Environ. Microbiol.*, 62:230–236.
47. Grimberg, S.J., Stringfellow, W.T., and Aitken, M.D., 1996, Quantifying the biodegradation of phenanthrene by *Pseudomonas stutzeri* P16 in the presence of a nonionic surfactant. *Appl. Environ. Microbiol.*, 62:2387–2392.
48. Grimm, A.C. and Harwood, C.S., 1999, NahY, a catabolic plasmid-encoded receptor required for chemotaxis of *Pseudomonas putida* to the aromatic hydrocarbon naphthalene. *J. Bacteriol.*, 181:3310–3316.
49. Grosser, R.J., Warshawsky, D., and Vestal, J.R., 1991, Indigenous and enhanced mineralization of pyrene, benzo[a]pyrene, and carboazole in soils. *Appl. Environ. Microbiol.*, 57:3462–3469.
50. Gschwend, P.M. and Hites, R.A., 1981, Fluxes of polycyclic aromatic hydrocarbons to marine and lacustrine sediments in the northeastern United States. *Geochim. Cosmochim. Acta*, 45:2359–2367.
51. Habe, H. and Omori, T., 2003, Genetics of polycyclic aromatic hydrocarbon metabolism in diverse aerobic bacteria. *Biosci. Biotechnol. Biochem.*, 67:225–243.
52. Harayama, S. 1997, Polycyclic aromatic hydrocarbon bioremediation design. *Curr. Opin. Biotechnol.*, 8:268–273.

53. Harayama, S. and Timmis, K.N., 1988, Catabolism of aromatic hydrocarbons by *Pseudomonas*. In D.A. Hopwood and K.F. Chater (eds), *Genetics of Bacterial Diversity*, pp. 151–174. Academic Press, New York.
54. Harayama, S., Kok, M., and Neidle, E.L., 1992, Functional and evolutionary relationships among diverse oxygenases. *Annu. Rev. Microbiol.*, 46:565–601.
55. Heitkamp, M.A. and Gerniglia, C.E., 1987, The effects of chemical structure and exposure on the microbial degradation of polycyclic aromatic hydrocarbons in freshwater and estuarine ecosystems. *Environ. Toxicol. Chem.*, 6:535–546.
56. Heitkamp, M.A., Freeman, J.P., Miller, D.W., and Cerniglia, C.E., 1988, Pyrene degradation by a *Mycobacterium* sp.: Identification of ring oxidation and ring fission products. *Appl. Environ. Microbiol.*, 54:2556–2565.
57. Herbes, S.E. and Schwall, L.R., 1978, Microbial transformation of polycyclic aromatic hydrocarbons in pristine and petroleum-contaminated sediments. *Appl. Environ. Microbiol.*, 35:306–316.
58. Hites, R.A., LaFlamme, R.E., and Farrington, J.W., 1977, Sedimentary polycyclic aromatic hydrocarbons: The historical record. *Science*, 198:829–831.
59. Hites, R.A., LaFlamme, R.E., and Windsor, J.G., 1980, Polycyclic aromatic hydrocarbons in marine/aquatic sediments: Their ubiquity. In L. Pertakis and E.T. Weiss (eds), *Petroleum in the Marine Environment*, pp. 289–311. Advances in Chemistry Series, American Chemical Society, Washington, DC.
60. Ho, Y., Jackson, M., Yang, Y., Mueller, J.G., and Pritchard, P.H., 2000, Characterization of fluoranthene- and pyrene-degrading bacteria isolated from PAH-contaminated soils and sediments. *J. Ind. Microbiol. Biotechnol.*, 24:100–112.
61. Holman, H.-Y.N., Tsang, Y.W., and Holman, W.R., 1999, Mineralization of sparsely water-soluble polycyclic aromatic hydrocarbons in a water table fluctuation zone. *Environ. Sci. Technol.*, 33:1819–1824.
62. Houghton, J.E. and Shanley, M.S., 1994, Catabolic potential of *Pseudomonads*: A regulatory perspective. In G.R. Chaudhry (ed.), *Biological Degradation and Bioremediation of Toxic Chemicals*, pp. 11–32. Kluwer, New York.
63. Huntley, S.L., Bonnevie, N.L., Wenning, R.J., and Bedbury, H., 1993, Distribution of polycyclic aromatic hydrocarbons (PAHs) in three northern New Jersey waterways. *Bull. Environ. Contam. Toxicol.*, 51:865–872.
64. Iwabuchi, T. and Harayama, S., 1997, Biochemical and genetic characterization of 2-carboxybenzaldehyde dehydrogenase, an enzyme involved in phenanthrene degradation by *Nocardioides* sp. strain KP7. *J. Bacteriol.*, 179:6488–6494.
65. Iwabuchi, T. and Harayama, S., 1998, Biochemical and molecular characterization of 1-hydroxy-2-naphthoate dioxygenase from *Nocardioides* sp. KP7. *J. Biol. Chem.*, 273:8332–8336.
66. Iwabuchi, T. and Harayama, S., 1998, Biochemical and genetic characterization of trans-2'-carboxybenzalpyruvate hydratase-aldolase from a phenanthrene-degrading *Nocardioides* strain. *J. Bacteriol.*, 180:945–949.
67. Jahan, K., Ahmed, T., and Maier, W.J., 1997, Factors affecting the nonionic surfactant enhanced biodegradation of phenanthrene. *Water Environ. Res.*, 69:317–325.
68. Johnson, A.C. and Larsen, D., 1985, The distribution of polycyclic aromatic hydrocarbons in the surficial sediments of Penobscot Bay (Maine, U.S.A.) in relation to possible sources and to other sites worldwide. *Mar. Environ. Res.*, 15:1–16.
69. Juhasz, A.L., Britz, M.L., and Stanley, G.A., 1997, Degradation of fluoranthene, pyrene, benz[a]anthracene and dibenz[a,h]anthracene by *Burkholderia cepacia*. *J. Appl. Microbiol.*, 83:189–198.
70. Juhasz, A.L., Stanley, G.A., and Britz, M.L., 2000, Microbial degradation and detoxification of high molecular weight polycyclic aromatic hydrocarbons by *Stenotrophomonas maltophilia* strain VUN 10,003. *Lett. Appl. Microbiol.*, 30:396–401.

71. Kanaly, R.A. and Harayama, S., 2000, Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. *J. Bacteriol.*, 182:2059–2067.
72. Kanaly, R.A., Harayama, S., and Watanabe, K., 2002, *Rhodanobacter* sp. strain BPC1 in a benzo[*a*]pyrene-mineralizing bacterial consortium. *Appl. Environ. Microbiol.*, 68:5826–5833.
73. Karimi-Lofabad, S., Pickard, M.A., and Gray, M.R., 1996, Reactions of polynuclear aromatic hydrocarbons on soil. *Environ. Sci. Tech.*, 30:1145–1151.
74. Kasai, Y., Inoue, J., and Harayama, S., 2001, The TOL plasmid pWW0 *xylN* gene product from *Pseudomonas putida* is involved in *m*-xylene uptake. *J. Bacteriol.*, 183:6662–6666.
75. Keith, L.H. and Telliard, W.A., 1979, Priority pollutants I—a perspective view. *Environ. Sci. Technol.*, 13:416–423.
76. Kelley, I. and Cerniglia, C.E., 1991, The metabolism of fluoranthene by species of *Mycobacterium*. *J. Ind. Microbiol.*, 7:19–26.
77. Kelley, I., Freeman, J.P., Evans, F.E., and Cerniglia, C.E., 1993, Identification of metabolites from the degradation of fluoranthene by *Mycobacterium* sp. strain PYR1. *Appl. Environ. Microbiol.*, 59:800–806.
78. Kelley, I., Freeman, J.P., Evans, F.E., and Cerniglia, C.E., 1991, Identification of a carboxylic acid metabolite from the catabolism of fluoranthene by *Mycobacterium* sp. *Appl. Environ. Microbiol.*, 57:636–641.
79. Kern, W., 1947, The occurrence of chrysene in soil. *Helv. Chim. Acta*, 30: 1595–1599.
80. Khan, A.A., Wang, R.F., Cao, W.W., Doerge, D.R., Wennerstrom, D., and Cerniglia, C.E., 2001, Molecular cloning, nucleotide sequence, and expression of genes encoding a polycyclic aromatic ring dioxygenase from *Mycobacterium* sp. strain PYR-1. *Appl. Environ. Microbiol.*, 67:3577–3585.
81. Kiyohara, H. and Nagao, K., 1978, The catabolism of phenanthrene and naphthalene by bacteria. *J. Gen. Microbiol.*, 105:69–75.
82. Kiyohara, H., Nagao, K., Kouno, K., and Yano, K., 1982, Phenanthrene-degrading phenotype of *Alcaligenes faecalis* AFK2. *Appl. Environ. Microbiol.*, 43:458–461.
83. Kiyohara, H., Torigoe, S., Kaida, N., Asaki, T., Iida, T., Hayashi, H., and Takigawa, N., 1994, Cloning and characterization of a chromosomal gene cluster, *pah*, that encodes the upper pathway for phenanthrene and naphthalene utilization by *Pseudomonas putida* OUS82. *J. Bacteriol.*, 176:2439–2443.
84. Kleespies, M., Kroppenstedt, R.M., Rainey, F.A., Webb, L.E., and Stackebrandt, E., 1996, *Mycobacterium holderi*, sp. nov., a new member of the fast-growing mycobacteria capable of degrading polycyclic aromatic hydrocarbons. *Int. J. Syst. Bacteriol.*, 46:683–687.
85. Koeber, R., Bayoma, J.M., and Niessner, R., 1999, Determination of benzo[*a*]pyrene diones in air particulate matter with liquid chromatography mass spectrometry. *Environ. Sci. Technol.*, 33:1552–1558.
86. Kurkela, S., Lehaslasio, H., Palva, E.T., and Terri, T.H., 1988, Cloning, nucleotide sequence and characterization of genes encoding naphthalene dioxygenase of *Pseudomonas putida* strain NCIB9816. *Gene*, 73:355–362.
87. Lamoureux, E.M. and Brownawell, B.J., 1999, Chemical and biological availability of sediment-sorbed hydrophobic organic contaminants. *Environ. Toxicol. Chem.*, 18:1733–1741.
88. Langworthy, D.E., Stapleton, R.D., Sayler, G.S., and Findlay, R.H., 1998, Genotypic and phenotypic responses of a riverine microbial community to polycyclic aromatic hydrocarbon contamination. *Appl. Environ. Microbiol.*, 64:3422–3428.
89. Laurie, A.D. and Lloyd-Jones, G., 1999, The *phn* genes of *Burkholderia* sp. strain RP007 constitute a divergent gene cluster for polycyclic aromatic hydrocarbon catabolism. *J. Bacteriol.*, 181:531–540.
90. Lee, S.D. and Grant, L., 1981, *Health and ecological assessment of polynuclear aromatic hydrocarbons*. Pathotox Publishers, Inc., Park Forest South, IL.

91. Lijinsky, W., 1991, The formation and occurrence of polynuclear aromatic hydrocarbons associated with food. *Mutat. Res.*, 259:251–261.
92. Lim, L.H., Harrison, R.M., and Harrad, S., 1999, The contribution of traffic to atmospheric concentrations of polycyclic aromatic hydrocarbons. *Environ. Sci. Technol.*, 33:3538–3542.
93. Martens, D., Maguhn, J., Spitzauer, P., and Kettrup, A., 1997, Occurrence and distribution of polycyclic aromatic hydrocarbons (PAHs) in an agricultural ecosystem. *Fresenius' J. Anal. Chem.*, 359:546–554.
94. Mason, J.R. and Cammack, R., 1992, The electron-transport proteins of hydroxylating bacterial dioxygenases. *Annu. Rev. Microbiol.*, 46:277–305.
95. Mastrangelo, G., Fadda, E., and Marzia, V., 1997, Polycyclic aromatic hydrocarbons and cancer in man. *Environ. Health Perspect.*, 104:1166–1170.
96. McNally, D.L., Miheleic, J.R., and Lueking, D.R., 1998, Biodegradation of three- and four-ring polycyclic aromatic hydrocarbons under aerobic and denitrifying conditions. *Environ. Sci. Technol.*, 32:2633–2639.
97. Means, J.C., Ward, S.G., Hassett, J.J., and Banwart, W.L., 1980, Sorption of polynuclear aromatic hydrocarbons by sediments and soils. *Environ. Sci. Technol.*, 14:1524–1528.
98. Meckenstock, R.U., Annweiler, E., Michaelis, W., Richnow, H.H., and Schink, B., 2000, Anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. *Appl. Environ. Microbiol.*, 66:2743–2747.
99. Miheleic, J.R. and Luthy, R.G., 1988, Degradation of polycyclic aromatic hydrocarbon compounds under various redox conditions in soil-water systems. *Appl. Environ. Microbiol.*, 54:1182–1187.
100. Moody, J.D., Freeman, J.P., Doerge, D.R., and Cerniglia, C.E., 2001, Degradation of phenanthrene and anthracene by cell suspensions of *Mycobacterium* sp. strain PYR-1. *Appl. Environ. Microbiol.*, 67:1476–1483.
101. Mueller, J.G., Chapman, P.J., Blattmann, B.O., and Pritchard, P.H., 1990, Isolation and characterization of a fluoranthene-utilizing strain of *Pseudomonas paucimobilis*. *Appl. Environ. Microbiol.*, 56:1079–1086.
102. Neu, T.R., 1996, Significance of bacterial surface-active compounds in interaction of bacteria with interfaces. *Microbiol. Rev.*, 60:151–166.
103. Ohkouchi, N., Kawamura, K., and Kawahata, H., 1999, Distributions of three- to seven-ring polynuclear aromatic hydrocarbons on the deep sea floor in the central Pacific. *Environ. Sci. Technol.*, 33:3086–3090.
104. Parales, R.E. and Harwood, C.S., 2002, Bacterial chemotaxis to pollutants and plant-derived aromatic molecules. *Curr. Opin. Microbiol.*, 5:266–273.
105. Patel, T.R. and Gibson, D.T., 1974, Purification and properties of (+)-*cis*-naphthalene dihydrodiol dehydrogenase of *Pseudomonas putida*. *J. Bacteriol.*, 119:879–888.
106. Pinyakong, O., Habe, H., and Omori, T., 2003, The unique aromatic catabolic genes in sphingomonads degrading polycyclic aromatic hydrocarbons (PAHs). *J. Gen. Appl. Microbiol.*, 49:1–19.
107. Pitt, R., Field, R., Llor, M., and Brown, M., 1995, Urban stormwater toxic pollutants: Assessment, sources and treatability. *Water Environ. Res.*, 67:260–275.
108. Prak, D.J. and Pritchard, P.H., 2002, Degradation of polycyclic aromatic hydrocarbons dissolved in Tween 80 surfactant solutions by *Sphingomonas paucimobilis* EPA 505. *Can. J. Microbiol.*, 48:151–158.
109. Rehmann, K., Hertkorn, N., and Kettrup, A.A., 2001, Fluoranthene metabolism in *Mycobacterium* sp. strain KR20: Identity of pathway intermediates during degradation and growth. *Microbiology*, 147:2783–2794.
110. Renner, R., 1999, EPA to strengthen persistent, bioaccumulative, and toxic pollutant controls—mercury first to be targeted. *Environ. Sci. Technol.*, 33:62A.

111. Rockne, K.J., Chee-Sanford, J.C., Sanford, R.A., Hedlund, B.P., Staley, J.T., and Strand, S.E., 2000, Anaerobic naphthalene degradation by microbial pure cultures under nitrate-reducing conditions. *Appl. Environ. Microbiol.*, 66:1595–1601.
112. Ron, E.Z. and Rosenberg, E., 2001, Natural roles of biosurfactants. *Environ. Microbiol.*, 3:229–236.
113. Ron, E.Z. and Rosenberg, E., 2002, Biosurfactants and oil bioremediation. *Curr. Opin. Biotechnol.*, 13:249–252.
114. Rothermich, M.M., Hayes, L.A., and Lovley, D.R., 2002, Anaerobic, sulfate-dependent degradation of polycyclic aromatic hydrocarbons in petroleum-contaminated harbor sediment. *Environ. Sci. Technol.*, 36:4811–4817.
115. Rouse, J.D., Sabatini, D.A., Suflita, J.M., and Harwell, J.H., 1994, Influence of surfactants on microbial degradation of organic compounds. *Crit. Rev. Environ. Technol.*, 24:325–370.
116. Saito, A., Iwabuchi, T., and Harayama, S., 1999, Characterization of genes for enzymes involved in the phenanthrene degradation in *Nocardioides* sp. KP7. *Chemosphere*, 38:1331–1337.
117. Saito, A., Iwabuchi, T., and Harayama, S., 2000, A novel phenanthrene dioxygenase from *Nocardioides* sp. strain KP7: Expression in *Escherichia coli*. *J. Bacteriol.*, 182:2134–2141.
118. Samanta, S.K., Chakraborti, A.K., and Jain, R.K., 1999, Degradation of phenanthrene by different bacteria: Evidence for novel transformation sequences involving the formation of 1-naphthol. *Appl. Microbiol. Biotechnol.*, 53:98–107.
119. Schneider, J., Grosser, R., Jayasimhulu, K., Xue, W., and Warshwsky, D., 1996, Degradation of pyrene, benz[a]anthracene, and benzo[a]pyrene by *Mycobacterium* sp. strain RJGII-135, isolated from a former coal gasification site. *Appl. Environ. Microbiol.*, 62:13–19.
120. Sepic, E., Bricej, M., and Leskovsek, H., 1998, Degradation of fluoranthene by *Pasteurella* sp. IFA and *Mycobacterium* sp. PYR1: Isolation and identification of metabolites. *J. Appl. Microbiol.*, 85:746–754.
121. Shuttleworth, K.L. and Cerniglia, C.E., 1995, Environmental aspects of PAH biodegradation. *Appl. Biochem. Biotechnol.*, 54:291–302.
122. Simon, M.J., Osslund, T.D., Saunders, R., Ensley, B.D., Suggs, S., Harcourt, A., Suen, W.-C., Cruden, D.L., Gibson, D.T., and Zylstra, G.J., 1993, Sequence of genes encoding naphthalene dioxygenase in *Pseudomonas putida* strains G7 and NCIB9816-4. *Gene*, 127:31–37.
123. Sims, J.L., Sims, R.C., and Matthews, J.E., 1990, Approach to bioremediation of contaminated soil. *Haz. Waste Haz. Mater.*, 7:117–149.
124. Sims, R.C. and Overcash, M.R., 1983, Polynuclear aromatic compounds (PNAs) in soil-plant systems. *Residue Rev.*, 88:1–68.
125. Smith, J.R., Nakles, D.V., Sherman, D.F., Neuhauser, E.F., and Loehr, R.C., 1989, Environmental fate mechanism influencing biological degradation of coal-tar derived polynuclear aromatic hydrocarbons in soil systems. In *The Third International Conference on New Frontiers for Hazardous Waste Management*, pp. 397–405. U.S. Environmental Protection Agency, Washington DC.
126. Smith, M.R., 1990, The biodegradation of aromatic hydrocarbons by bacteria. *Biodegradation*, 1:191–206.
127. Story, S.P., Parker, S.H., Kline, J.D., Tzeng, T.R., Mueller, J.G., and Kline, E.L., 2000, Identification of four structural genes and two putative promoters necessary for utilization of naphthalene, phenanthrene, fluoranthene by *Sphingomonas paucimobilis* var. EPA505. *Gene*, 260:155–169.
128. Takizawa, N., Kaida, N., Torigoe, S., Moritani, T., Sawada, T., Satoh, S., and Kiyohara, H., 1994, Identification and characterization of genes encoding polycyclic aromatic hydrocarbon dioxygenase and polycyclic aromatic hydrocarbon dihydrodiol dehydrogenase in *Pseudomonas putida* OUS82. *J. Bacteriol.*, 176:2444–2449.

129. Tiehm, A., 1994, Degradation of polycyclic aromatic hydrocarbons in the presence of synthetic surfactants. *Appl. Environ. Microbiol.*, 60:258–263.
130. Treadway, S.L., Yamagimachi, K.S., Lankenau, E., Lessard, P.A., Stephanopoulos, G., and Sinskey, A.J., 1999, Isolation and characterization of indene bioconversion genes from *Rhodococcus* strain I24. *Appl. Microbiol. Biotechnol.*, 51:786–793.
131. Tsuda, M. and Iino, T., 1990, Naphthalene degrading genes on plasmid NAH7 are on a defective transposon. *Mol. Gen. Genet.*, 223:33–39.
132. van Brummelen, T.C., Verweij, R.A., Wedzinga, S.A., and van Gestel, C.A.M., 1996, Enrichment of polycyclic aromatic hydrocarbons in forest soils near a blast furnace plant. *Chemosphere*, 32:293–314.
133. van der Meer, J.R., de Vos, W.M., Harayama, S., and Zehnder, J.B., 1992, Molecular mechanisms of genetic adaptation to xenobiotic compounds. *Microbiol. Rev.*, 56:677–694.
134. Vila, J., Lopez, Z., Sabate, J., Minguillon, C., Solanas, A.M., and Grifoll, M., 2001, Identification of a novel metabolite in the degradation of pyrene by *Mycobacterium* sp. strain AP1: Actions of the isolate on two- and three-ring polycyclic aromatic hydrocarbons. *Appl. Environ. Microbiol.*, 67:5497–5505.
135. Volkerling, F., Breure, A.M., Sterkenburg, A., and van Andel, J.G., 1992, Microbial degradation of polycyclic aromatic hydrocarbons: Effect of substrate availability on bacterial growth kinetics. *Appl. Microbiol. Biotechnol.*, 36:548–552.
136. Wagrowski, D.M. and Hites, R.A., 1997, Polycyclic aromatic hydrocarbon accumulation in urban, suburban, and rural vegetation. *Environ. Sci. Technol.*, 31:279–282.
137. Walter, U., Beyer, M., Klein, J., and Rehm, H.-J., 1991, Degradation of pyrene by *Rhodococcus* sp. UW1. *Appl. Microbiol. Biotechnol.*, 34:671–676.
138. Weis, L.M., Rummel, A.M., Masten, S.J., Trosko, J.E., and Upham, B.L., 1998, Bay and bay-like regions of polycyclic aromatic hydrocarbons were potent inhibitors of gap junctional intercellular communication. *Environ. Health Perspect.*, 106:17–22.
139. Weissenfels, W.D., Beyer, M., and Klein, J., 1990, Degradation of phenanthrene, fluorene and fluoranthene by pure bacterial cultures. *Appl. Microbiol. Biotechnol.*, 32:479–484.
140. Whitman, B.E., Lueking, D.R., and Mihelcic, J.R., 1998, Naphthalene uptake by a *Pseudomonas fluorescens* isolate. *Can. J. Microbiol.*, 44:1086–1093.
141. Wick, L.Y., de Munain, A.R., Springael, D., and Harms, H., 2002, Responses of *Mycobacterium* sp. LB501T to the low bioavailability of solid anthracene. *Appl. Microbiol. Biotechnol.*, 58:378–385.
142. Williams, P.A. and Sayers, J.R., 1994, The evolution of pathways for aromatic hydrocarbon oxidation in *Pseudomonas*. *Biodegradation*, 5:195–217.
143. Willumsen, P.A. and Arvin, E., 1999, Kinetics of degradation of surfactant-solubilized fluoranthene by a *Sphingomonas paucimobilis*. *Environ. Sci. Technol.*, 33:2571–2578.
144. Wild, S.R., Obbard, J.P., Munn, C.I., Berrow, M.L., and Jones, K.C., 1991, The long-term persistence of polynuclear aromatic hydrocarbons (PAHs) in an agricultural soil amended with metal-contaminated sewage sludges. *Sci. Total Environ.*, 101:235–253.
145. Yang, Y., Chen, R.F., and Shiaris, M.P., 1994, Metabolism of naphthalene, fluorene, and phenanthrene: Preliminary characterization of a cloned gene cluster from *Pseudomonas putida* NCIB 9816. *J. Bacteriol.*, 176:2158–2164.
146. Yen, K.M. and Gunsalus, I.C., 1982, Plasmid gene organization: Naphthalene/salicylate oxidation. *Proc. Natl. Acad. Sci. USA*, 79:874–878.
147. Yen, K.M. and Serdar, C.M., 1988, Genetics of naphthalene catabolism in pseudomonads. *Crit. Rev. Microbiol.*, 15:247–268.
148. Zeng, E. and Vista, C.L., 1997, Organic pollutants in the coastal environment off San Diego, California. 1. Source identification and assessment by compositional indices of polycyclic aromatic hydrocarbons. *Environ. Toxicol. Chem.*, 16:179–188.

149. Zhang, X. and Young, L.Y., 1997, Carboxylation as an initial reaction in the anaerobic metabolism of naphthalene and phenanthrene by sulfidogenic consortia. *Appl. Environ. Microbiol.*, 63:4759–4764.
150. Zhou, N.-Y., Fuenmayor, S.L., and Williams, P.A., 2001, *nag* genes of *Ralstonia* (formerly *Pseudomonas*) sp. strain U2 encoding enzymes for gentisate catabolism. *J. Bacteriol.*, 183:700–708.
151. Zylstra, G.J. and Gibson, D.T., 1991, Aromatic hydrocarbon degradation. In J.K. Setlow (ed.), *A Molecular Approach in Genetic Engineering: Principles and Methods*, vol. 13, pp. 183–203. Plenum Press, New York.

OVEREXPRESSION AND SECRETION OF *PSEUDOMONAS* LIPASES

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1. INTRODUCTION

Enzymes are biocatalysts which catalyze a given reaction in a living cell with high activity, substrate specificity and regio- as well as enantioselectivity. They work at ambient temperatures and pressures, and several biocatalysts function in aqueous environments, but also in organic solvents. Therefore, the chemical industry nowadays uses biocatalysts for hydrolytic and synthetic reactions to produce complex drug intermediates, speciality or commodity chemicals, and, most importantly, enantiopure pharmaceuticals. The number of biocatalytic processes installed to replace energy-consuming and toxic waste producing chemical processes is rapidly increasing⁷¹ thereby creating a rapidly growing demand to identify and produce biocatalysts with novel and biotechnologically useful properties. To achieve this, novel technologies have recently been developed: (a) environmental DNA, the so-called metagenome, is directly cloned and assayed for the presence of novel biocatalytic activities thereby circumventing the need to cultivate novel microorganisms⁴⁹; (b) directed evolution and new methods for protein engineering enable the optimization of existing biocatalysts^{5, 38, 57}. However, all these approaches rely on efficient systems for the overexpression as well as large scale and often heterologous production of biocatalyst proteins. *Escherichia coli* is still the most widely used host strain for

all kinds of molecular genetic manipulations including expression of heterologous genes, and several approaches were published to produce heterologous proteins in different extracytoplasmic compartments of *E. coli*¹⁹, but the production of enzymatically active enzymes in *E. coli* is often difficult or even impossible. This is particularly true for extracellular enzymes, the major reason being that *E. coli* itself does not normally secrete enzymes into the extracellular growth medium. In contrast, bacteria belonging to the genus *Pseudomonas* are known to produce and secrete a wealth of extracellular enzymes. Therefore, this group of bacteria has recently emerged as a reasonable alternative for the expression and secretion of biocatalyst proteins¹² with *Pseudomonas aeruginosa* being the best studied model organism.

Among the enzymes used in biotechnology and synthetic organic chemistry, lipases constitute the most important group. Interestingly, lipases originating from several *Pseudomonas* and *Burkholderia* species seem to be particularly useful for a wide variety of different biocatalytic applications including the enantioselective production of alcohols, amines, and carbonic acids^{33, 35, 40, 48, 64}. The production of these lipases requires not only the efficient overexpression of the corresponding genes but also a detailed understanding of the molecular mechanisms governing their folding and secretion. In fact, *Pseudomonas* lipases require the functional assistance of about 30 different cellular proteins before they can be recovered from the culture supernatant in an enzymatically active state indicating that folding and secretion are highly specific processes which normally do not function properly in heterologous hosts⁶⁶.

2. FOLDING AND SECRETION OF *PSEUDOMONAS* LIPASES

Lipases which are secreted into the culture supernatant are preferred for many biotechnological applications. Chemically, the supernatant resembles the growth medium and usually does not contain too many different and competing enzyme activities as compared to a whole cell lysate thereby making enzyme activity assays much easier to perform. Furthermore, the subsequent down-stream processing for the isolation and purification of lipases also becomes cheaper and more effective when extracellular enzymes can be used.

2.1. Pathways for Lipase Secretion

Lipases from *Pseudomonas* and *Burkholderia* have been classified into three distinct subfamilies of lipase family I⁶. Prototype lipases of the subfamilies I.1 and I.2 are those from *P. aeruginosa* and *Burkholderia glumae* which have an

average M_r of 30 kDa and share a high degree of sequence homology as well as several common physiological features including their mechanisms of secretion. The prototype enzyme of subfamily I.3 is the *Pseudomonas fluorescens* lipase which is larger (M_r : 50 kDa) and also uses a different secretion pathway^{32, 66}.

2.1.1. One-Step Secretion

Secretion via ABC protein exporters^{1, 42} occurs in one-step by translocation of proteins directly from the cytoplasm to the extracellular medium without the formation of periplasmic intermediates. Lipases from *P. fluorescens* strains no.33 and SIK W1 as well as from *Serratia marcescens*³ and *Pseudomonas brassicacearum*¹⁶ are secreted by this pathway. These enzymes lack an N-terminal signal sequence but contain C-terminal targeting signals which are essential for the recognition by the ABC exporters^{11, 20} (Figure 1). The one-step or type I secretion machinery consists of three proteins: an integral outer membrane protein, an inner membrane protein belonging to the membrane fusion protein (MFP) family, and an ABC-transporter protein which belongs to the ATP-binding cassette superfamily of eukaryotic and prokaryotic protein exporters⁵⁸. Recently, the crystal structure of the outer membrane transporter TolC from *E. coli* was solved. This protein contains a single hydrophilic pore formed by a trimeric complex in the outer membrane and α -helical appendages which build a tunnel spanning the periplasmic space⁴. The protein components of different ABC-transporters can be interchanged between different bacterial species, at least to a certain extent² and the specificity of the interaction between a given transporter complex and its cognate target proteins seems to be restricted to the carboxy-terminal targeting signals. In addition, the *P. aeruginosa* and *P. fluorescens* genomes encode four and three different type I secretion systems, respectively, some of which naturally secrete lipases⁵⁰. Consequently, it should in principle be possible to engineer *Pseudomonas* strains for an ABC-transporter-mediated secretion of heterologous lipases.

2.1.2. Two-Step Secretion

The two-step secretion pathway includes the initial translocation of the secreted protein across the inner membrane to form a periplasmic intermediate, and finally its transport through the outer membrane. At present, two pathways each were described for secretion through the inner and the outer membrane, respectively.

2.1.2.1. Secretion Across the Inner Membrane. Most of the exoproteins in Gram-positive and in Gram-negative bacteria harbor N-terminal signal peptides which constitute specific targeting signals directing the precursors proteins to

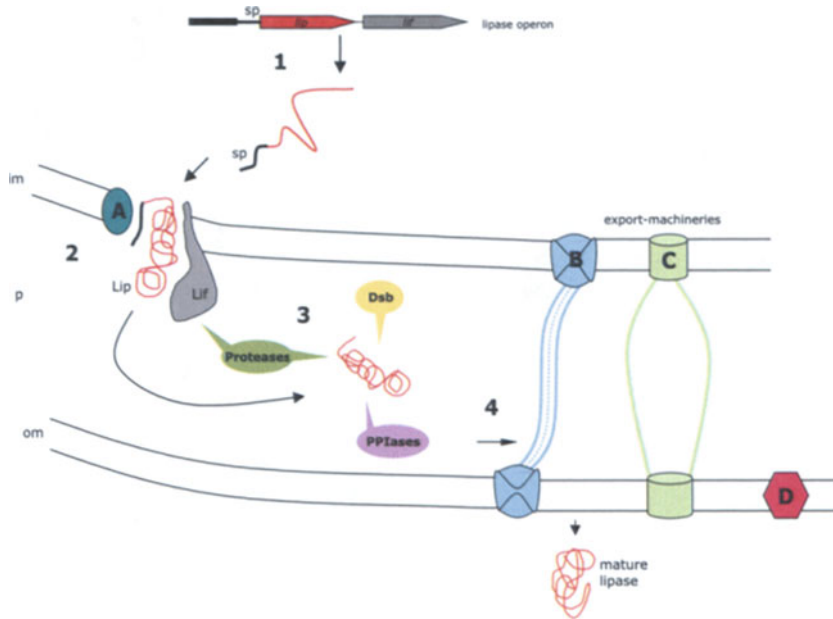


Figure 1. The production of enzymatically active biocatalysts which are secreted to the extracellular space requires four distinct steps which form potential bottlenecks of this process. 1. The structural gene has to be efficiently expressed. 2. The newly synthesized protein is translocated across the inner membrane (im) by either the Sec-machinery or the Tat-exporter system (A). 3. Periplasmic folding requires the assistance of accessory proteins. In the case of the lipase (Lip), the action of the specific chaperone (Lrf) and of additional folding catalysts including Dsb proteins which catalyze the formation of disulphide bonds is essential. In addition, periplasmic PPases probably serve as folding catalysts. The periplasmic folding process is influenced by proteases which either degrade misfolded proteins or control the concentration of specific folding catalysts. 4. Correctly folded biocatalyst proteins are subsequently recognized by protein exporters governing the transport across the outer membrane (om). Family I.1 and I.2 lipases use the type II secretion pathway (B) to reach their final destination, whereas family I.3 lipases bypass the periplasmic folding compartment due to their secretion by the ABC exporter pathway (C). Lipolytic enzymes of the EstA type possess an autotransporter domain which itself forms a secretion pore in the outer membrane through which the enzymatically active domain is translocated (D).

the Sec-machinery which is a multisubunit complex consisting of the inner membrane proteins SecY, E, D, G, and F¹⁵. For many years, the Sec-machinery was regarded as being unique in its ability to translocate bacterial proteins into the periplasm and homologues of the *E. coli* *sec*-genes have been identified in every known bacterial genome of both Gram-negative and Gram-positive species demonstrating the universal character of this secretion mechanism. Most of the exoproteins of *P. aeruginosa* including the lipase LipA also use this mechanism for their passage through the inner membrane into the periplasm.

Recently, a second secretion pathway has been identified in *E. coli* which also mediates the translocation of proteins across the inner membrane^{9, 10}. Proteins using this novel pathway are also synthesized as precursors with cleavable N-terminal signal peptides, which contain a characteristic doublet of conserved arginine residues being essential for the recognition by this export machinery. Therefore, this pathway was named Tat for *twin-arginine-translocation*. Interestingly, in contrast to the Sec-system, the Tat-pathway mediates the secretion of proteins which are already folded prior to their secretion^{9, 10}. Recent findings show that a functional Tat-secretion system also exists in *P. aeruginosa*⁷⁷. An elegant study revealed that the inactivation of the Tat machinery resulted in a *P. aeruginosa* strain which showed a severely reduced virulence in a rat lung infection model⁵⁴. Apart from several periplasmic proteins, the phospholipases PlcH and PlcN were found to be translocated via the Tat-pathway through the inner membrane indicating that this pathway is also used for the secretion of extracellular enzymes^{54, 77}. Recent results indicate that heterologous proteins may be directed into the periplasm via the Tat- but not the Sec-pathway as shown in *E. coli* for the green fluorescent protein GFP from *Aequoria victoria*⁸. Productive folding of GFP requires the assistance of cytoplasmic chaperones which cannot be replaced by periplasmic folding catalysts. These findings offer the opportunity to secrete even those heterologous proteins into the periplasm which need to be folded in the cytoplasm. In addition, *Pseudomonas* strains provide several secretion mechanisms to translocate periplasmic proteins through the outer membrane which may facilitate the creation of a general *Pseudomonas* secretion host.

2.1.2.2. Secretion Across the Outer Membrane. *Pseudomonas aeruginosa* produces three lipolytic enzymes, the extracellular lipases LipA and LipC, and the esterase EstA which is located in the bacterial outer membrane⁷⁹. The N-terminal domain of EstA contains a GDSL active site consensus motif typical for a novel class of lipolytic enzymes⁷⁵, whereas the C-terminal domain shows homology to the β - or transporter domain of autotransporter proteins⁷⁹, which usually consists of 14 β -sheets forming a pore-like β -barrel inserted in the outer membrane through which the N-terminal catalytic α -domain is translocated⁸¹. In contrast to other autotransporter proteins, the N-terminal domain of EstA remains attached to the C-terminal domain even after translocation through the outer membrane thereby anchoring the catalytic activity to the cell surface⁷⁹.

Burkholderia and *Pseudomonas* lipases belonging to subfamilies I.1 and I.2 fold in the periplasm into an enzymatically active conformation and are subsequently transported through the outer membrane by means of a complex machinery consisting of up to 14 different proteins. In *P. aeruginosa*, this type II secretion machinery or 'secreton' is built from 12 Xcp-proteins and is

required for the outer membrane translocation of several exoenzymes, among them exotoxin A, phospholipases C, elastase LasB, and the lipases LipA and LipC^{24, 51}, which can be delivered into the periplasm either by the Sec- or by the Tat-pathway⁷⁷. So far, experimental indications for a limited secretion capacity of the type II machinery are lacking; however, secretion of the extracellular lipase from *Pseudomonas alcaligenes* could be significantly improved when additional copies of the genes encoding the type II secretion machinery were coexpressed together with the lipase²⁷. A more general use of type II secretion, for example, for the overproduction of biocatalysts is hampered by the fact that a specific recognition of the secreted enzymes by the export machinery is required but the recognition signals have not yet been identified⁶⁸.

2.2. Periplasmic Folding Catalysts

The folding of lipases requires specific as well as non-specific folding catalysts several of which have recently been identified and characterized. The most prominent specific intermolecular folding catalysts are designated 'lipase-specific foldases' or Lif proteins³². These foldases are usually encoded in the same operon together with their cognate lipases and have been identified in several *Pseudomonas* strains^{17, 30, 31, 43, 56, 80}, *Burkholderia cepacia*⁴¹, *B. glumae*^{25, 26}, *Acinetobacter calcoaceticus*^{44, 74} and *Vibrio cholerae*⁵⁵. The lipase LipC of *P. aeruginosa* represents an exception in that it needs for folding a Lif protein which is encoded in a different operon together with the lipase LipA⁵¹ (F. Rosenau, G. Friedrich and K.-E. Jaeger, unpublished results). According to present knowledge, Lif proteins provide steric information for the folding process of lipases and have therefore been designated 'steric chaperones'²².

Periplasmic folding requires not only enzyme-specific inter- or intramolecular chaperones but also general folding catalysts. Proteins secreted by the type II pathway often contain disulfide bonds which are formed in the periplasm by the so-called Dsb proteins involved in disulfide bond formation. These proteins which were first identified and extensively characterized in *E. coli*¹⁸ are known to be conserved in Gram-negative bacteria. In *P. aeruginosa*, the thiol:disulfide oxidoreductase DsbA is needed for the maturation of several exoproteins, among them alkaline phosphatase (A. Urban *et al.*, unpublished results), elastase¹³, and lipase^{47, 76}. Inactivation of the *dsbA* gene in *P. aeruginosa* resulted in a reduction by 90% of the extracellular lipase production which was further reduced to 1% residual activity in a *dsbA dsbC* double mutant. The *dsbC* structural gene encodes a thiol:disulfide isomerase which is also involved in the re-oxidation of DsbA. Interestingly, the amount of extracellular lipase increased by a factor of two upon inactivation of the *dsbC* gene⁷⁶.

Peptidyl-prolyl-*cis/trans* isomerases (PPIases) represent another important class of general folding catalysts. They catalyze the *cis-trans* isomerization

of peptide bonds of the peptide backbone, usually at proline residues^{28, 69}. Interestingly, lipases from *P. aeruginosa* and *B. glumae*⁵² also contain *cis*-peptide bonds, here connecting residues Q257-V258 and Q291-L292, respectively. At present, a physiological or structural function of this *cis*-bond is unknown as is the mechanism or type of enzyme involved in their formation.

2.3. Periplasmic Events Determine the Overexpression Efficiency

A detailed understanding of the cellular processes governing folding and secretion of lipases is a necessary prerequisite for the creation of a potent bacterial secretion host strain and the periplasm represents the most important cellular compartment for these processes. Here, most of the extra-cytoplasmic proteins are folded into their native conformation, and sophisticated mechanisms exist to detect and degrade misfolded proteins⁵⁹. The lipases from *P. aeruginosa* and *B. glumae* are folded during or after translocation into the periplasm by their cognate Lif foldases. Recently, it was demonstrated that the simple overexpression of the respective Lif proteins in a wild-type background resulted in a dramatic increase of lipase production and secretion indicating that the expression level of the chaperone is limiting for lipase production under physiological conditions although both proteins are usually coexpressed from the same operon²³. This genetic organization suggests a close coupling between lipase and Lif expression; however, in contrast to the lipase, the Lif protein is hardly detectable in wild-type *P. aeruginosa* cells even by immunological methods (see Figure 2B). This result indicates that Lif's underlie a rapid turnover within the cells, presumably taking place in the periplasm. Recently, we have identified in the *P. aeruginosa* genome (available at www.pseudomonas.com), seven genes which share significant homologies with periplasmic proteases of different Gram-negative bacteria. These genes were inactivated and the resulting mutant strains were analyzed for lipase production. The mutant strain lacking a periplasmic protease homologous to the *E. coli* CtpA protease showed a significantly increased extracellular lipase activity and also a drastically increased cellular amount of Lif protein (Figure 2). Thus, a decreased turnover rate of the Lif foldase seems to result in an increased production of lipase thereby overcoming a limitation of the folding capacity under physiological conditions within the periplasm. In addition to this CtpA-like protease, the genome sequence of *P. aeruginosa* contains a number of open reading frames encoding other putative periplasmic chaperones or folding catalysts. We have started to investigate their role for the production of extracellular enzymes in *P. aeruginosa*.

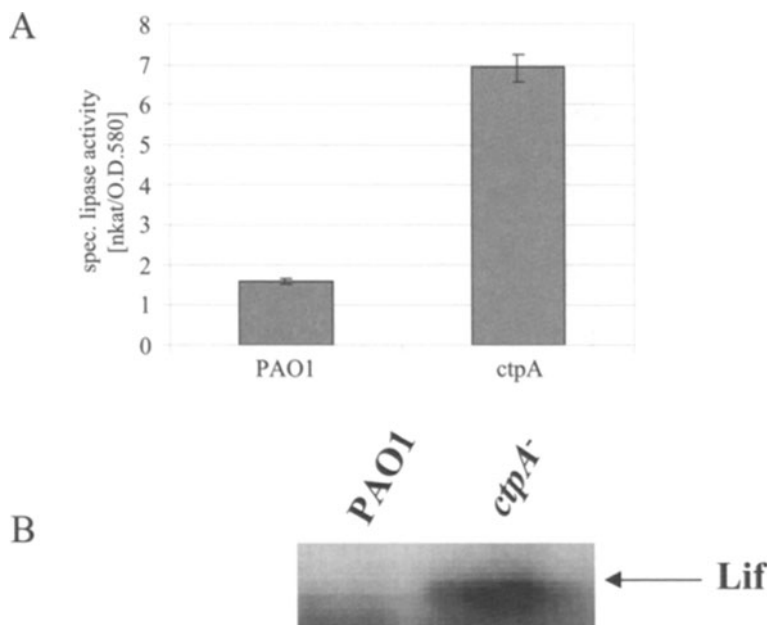


Figure 2. The periplasmic protease CtpA significantly influences the yield of extracellular lipase by controlling the cellular concentration of Lif. The lipase operon was expressed in the wild-type strain *P. aeruginosa* PAO1 and in a mutant strain carrying a deletion in the *ctpA* gene encoding the periplasmic protease CtpA. A. Extracellular lipase activity was assayed spectrophotometrically using p-nitrophenylpalmitate as a substrate. Lipase activity was normalized to the cell density of the culture measured as the optical density at 580 nm (O.D.₅₈₀). B. Western blot of whole cell lysates using an anti-Lif polyclonal antiserum.

3. *PSEUDOMONAS* LIPASES FOR BIOTECHNOLOGICAL APPLICATIONS

The commercial use of lipases comprises a wide variety of different applications in the areas of detergents, in the production of food ingredients, and enantiopure pharmaceuticals^{35, 36, 70}. Therefore, a strong pressure exists to (a) identify and isolate novel lipase genes, and (b) optimize existing lipases with respect to desired properties. Genomics, the exploitation of biodiversity, and directed enzyme evolution serve to provide a wealth of novel genes⁷² which have to be overexpressed and produced to make the corresponding enzymes available to the biotechnology industry.

3.1. Overexpression of *Pseudomonas* Lipases

Overexpression first requires the efficient transcription of a target gene. However, the production of a high transcript quantity itself does not always guarantee the high level production of the respective protein, since it is often accompanied by misfolding and intracellular deposition of the protein in the form of insoluble inclusion bodies. In *E. coli*-based systems, inducible T7-RNA polymerase has successfully been used to increase the expression levels of many different target genes⁷ with the pET-vector series (commercialized by Novagen, Madison, USA), representing the best known system. However, a considerable number of biocatalyst proteins cannot be expressed using this or a related system because their production requires several accessory cellular functions. Some enzymes may need essential cofactors for activity and unique chaperones for folding. Moreover, secretion may also be essential to achieve an enzymatically active state of the enzyme. Therefore, the development of an efficient system allowing for overexpression in combination with subsequent secretion is not trivial and requires a detailed knowledge of the biocatalyst protein, its biochemical properties, and the cellular pathway involved in its biosynthesis, folding, and secretion. A suitable host strain fulfilling all these requirements needs to be defined which should also allow the transformation of foreign DNA with high efficiency. Finally, to make the system useful for directed evolution experiments, the expression level must be high enough for high throughput screening which is usually performed in microtiter plates (culture volume: 10–100 μ l) after growth of the cultures for several hours.

3.1.1. Overexpression Systems

The T7-overexpression system originally developed for *E. coli* has also been adapted to construct *P. aeruginosa* overexpression strains^{14, 29, 34, 45, 73, 78}. Brunshwig and Darzins have constructed the *P. aeruginosa* strain PADD 1976 which contained a cassette composed of the T7-RNA polymerase structural gene under control of a *lacUV5* promoter and, in addition, the *lacI^q* gene encoding the *E. coli* Lac repressor¹⁴. Initial experiments demonstrated that this strain was a suitable host strain for the overexpression of *P. aeruginosa* lipase³⁴. However, it still harbored the wild-type lipase gene and therefore produced a significant background lipase activity. Therefore, we have constructed *P. aeruginosa* PABST7.1 which carries a large deletion in the lipase operon covering about 600 bp of the lipase structural gene *lipA* and 300 bp of the foldase gene *lif*. Additionally, *P. aeruginosa* PAFRT7.7 was constructed by site-specific integration of the expression cassette into the lipase operon³⁴ resulting in a lipase-negative mutant suitable for overexpression of lipase as well as for the background-free expression of mutant lipase genes constructed in directed

evolution experiments. Both *P. aeruginosa* strains produced about 150 mg of lipase per liter of culture supernatant.

Recently, we have constructed the expression vector pBBR22b which is based on the mobilizable broad host range vector pBBR1MCS⁴⁵. This novel vector is suitable for the overexpression of different target genes in strains other than *E. coli*. It harbors in combination a T7 promoter (P_{T7}), a *lac*-operator (*lacO*), and a strong ribosomal binding site (SD). Furthermore, in-frame fusions with the *pelB* signal sequence allow for a Sec-dependent translocation of overexpressed target proteins into the periplasm of several different host cells and a His-tag coding sequence enables the construction of target proteins which can be purified by a one-step affinity chromatography.

The novel vector pBBR22b⁶⁷ in combination with *P. aeruginosa* strain PABST7.1 was used to express (a) the lipase LipA and its variants for subsequent purification and 3D-structure determination; (b) the outer membrane esterase EstA and the lipase LipC for immunization and biochemical characterization; and (c) a His-tagged variant of the *P. aeruginosa* response regulator protein CbrB⁵³ (formerly named LipR) for in vitro DNA-binding studies. In all these cases, an approximate yield in the range of 5–10 mg protein per liter of culture was obtained without any optimization of the expression or growth conditions (F. Rosenau *et al.*, in preparation). The *P. aeruginosa* PABST7.1-pBBR22b system also allowed the overexpression of a lipase from *Arabidopsis thaliana* with high efficiency reaching yields of 10–20% of total cellular protein (M. Juenger *et al.*, unpublished results) thereby demonstrating that *P. aeruginosa* can serve as an efficient expression host for the high yield production of plant proteins.

3.1.2. Optimization of Lipase Folding in *Pseudomonas*

Lipase-specific foldases (Lifs) are highly specific for their cognate lipases²¹ thereby posing severe limitations to the creation of novel lipases, for example by directed evolution, because their structures may differ too much from the corresponding wild-type progenitors to be recognized by the corresponding Lif proteins. A solution to this problem may evolve from the recent finding of a lipase variant from *Pseudomonas* species KFCC 10818 which carried just the single amino acid exchange P112Q and folded into its active conformation displaying 63% of the wild-type enzymatic activity even in the absence of its cognate Lif protein⁴³. If confirmed with other Lif-dependent lipases this result may have important consequences for the construction of novel high-yield production host strains.

We have investigated in more detail the specificity of the lipase–Lif interactions in different *Pseudomonas* and *Burkholderia* species by cloning a collection of several lipase and Lif genes into the expression plasmid pBR22b. This ‘toolbox’ of lipase and foldase genes allows their combinatorial cloning

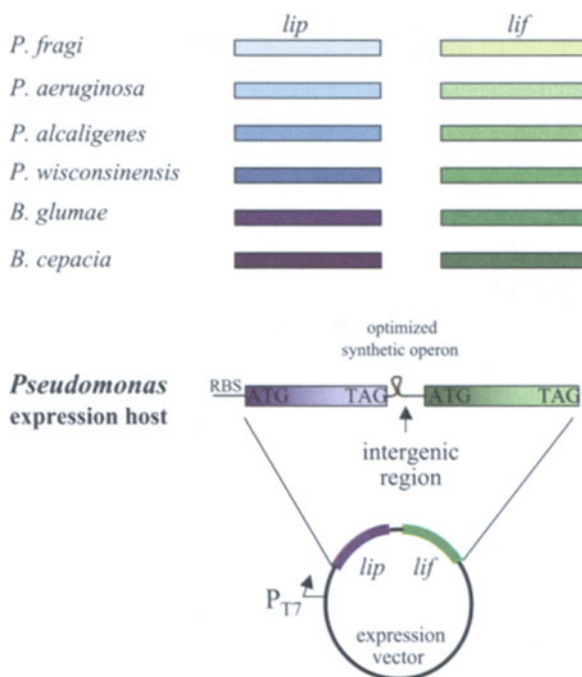


Figure 3. Structural genes (*lip* and *lif*) encoding lipases and Lif proteins from different *Pseudomonas* and *Burkholderia* species were cloned separately into the expression vector pBBR22b under transcriptional control of the strong T7 promoter. This collection of identically cloned genes allows their combination in synthetic operon structures suitable for expression in *P. aeruginosa* and other Gram-negative host strains providing the T7-RNA polymerase.

to create a variety of synthetic lipase operons (Figure 3). Preliminary results obtained by coexpression of *lif* and heterologous *lip* genes in various combinations indicated that an efficient folding of lipases can only be achieved by Lif proteins originating from closely related species. The Lif of *P. alcaligenes* can catalyze the folding of *P. aeruginosa* lipase, at least to a certain extent²¹. We have found that the expression in *P. aeruginosa* of a synthetic operon consisting of the *P. aeruginosa* lipase and *P. alcaligenes* foldase genes resulted in an efficient lipase secretion. The *lip* and *lif* genes from *P. aeruginosa* and *Pseudomonas wisconsinensis* were also functionally exchangeable, whereas the lipases from *B. glumae* and *B. cepacia* could not be activated by Lif proteins from *P. aeruginosa*, *P. alcaligenes*, or *P. wisconsinensis* (F. Rosenau, D. Janosch and K.-E. Jaeger, unpublished results). Further studies of Lif proteins which are essential to achieve enzymatic activity and secretion of lipases will hopefully allow the construction of a 'general' Lif protein which can catalyze the activation of different lipases even if they are distantly related.

3.2. Creation of Enantioselective *P. aeruginosa* Lipase by Directed Evolution

The steadily growing requirement for enantiopure compounds to be produced by the chemical and pharmaceutical industries forces the development of novel methods to create enantioselective biocatalysts. The first and most comprehensive study to create an enantioselective enzyme by directed evolution was performed with the lipase LipA from *P. aeruginosa*^{35, 37–39, 46, 60–62, 65}. The model reaction was the hydrolytic kinetic resolution of the chiral 2-methyldecanoic acid *p*-nitrophenyl ester which is catalyzed by *P. aeruginosa* lipase with an enantioselectivity of only *ee* 2% in favor of the *S*-ester at about 50% conversion corresponding to a selectivity factor *E* of 1.1. After four rounds of mutagenesis using ep-PCR at a low mutagenesis rate and spectrophotometric screening in microtiter plates, several lipase variants were identified with enantioselectivities increased to *ee* = 81% (*E* = 11). A significant further increase was obtained by combining ep-PCR with saturation and site-directed mutagenesis at those positions at which amino acid substitution had occurred ('hot spots'). Thus, several variants were obtained which displayed *ee*-values of 90–94% (*E* = 20–25) with the best variant containing

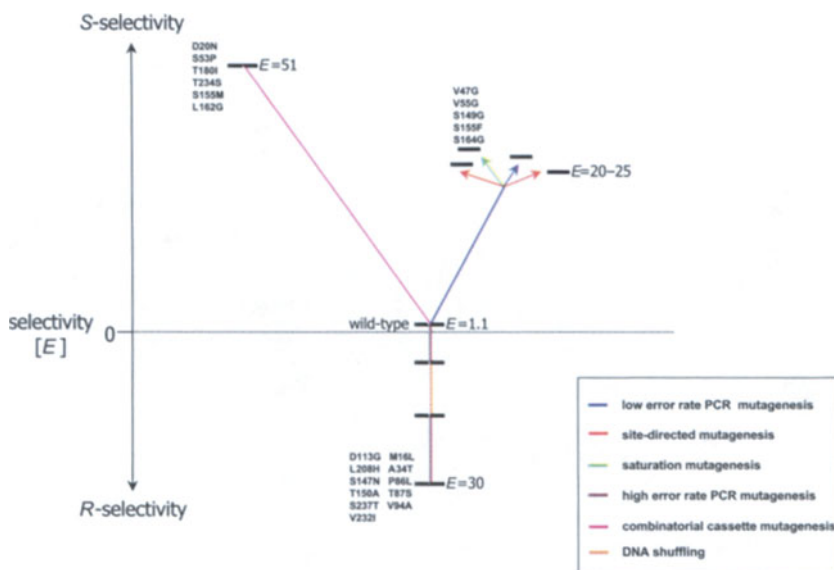


Figure 4. Creation of enantioselective *P. aeruginosa* lipases by employing different directed evolution methods which are indicated by colored lines. Amino acid substitutions of the best (*S*)- and (*R*)-selective variants are named.

five mutations V47G, V55G, S149G, S155F, and S164G⁴⁶. In addition, multiple combinatorial cassette mutagenesis was used to further increase the enantioselectivity of LipA. A cassette was isolated and saturated with mutations at positions corresponding to amino acids 155 and 162 which had previously been identified as being important for the enantioselectivity of *P. aeruginosa* lipase. This saturation library was recombined by DNA shuffling with libraries isolated by high error rate PCR of the entire LipA gene resulting in a highly *S*-selective variant ($E = 51$) that contains six amino acid substitutions D20N, S53P, S155M, L162G, T180I, T234S⁶³ (Figure 4).

The reversion of an enzyme's enantioselectivity constitutes another challenge. Starting from the first generation libraries described above, we used high-error-rate PCR mutagenesis to create (*R*)-selective variants which were further improved by DNA shuffling (Figure 4). An appropriate combination of ep-PCR at high error rate and DNA shuffling finally led us to identify a highly (*R*)-selective variant ($E = 30$) that carries 11 amino acid substitutions M16L, A34T, P86L, T87S, V94A, D113G, S147N, T150A, L208H, V232I, and S237T⁸².

4. CONCLUSIONS

Many bacteria are known to produce and often secrete enzymes which are increasingly used for biotechnological applications because they exhibit interesting and sometimes unique biochemical properties. Additionally, a growing number of novel biocatalyst genes are currently being identified from novel genome sequences and metagenomic DNA, or generated by techniques of directed evolution. How can the potential biotechnological value of all these genes and enzymes be assessed? Efficient overexpression systems and suitable host strains are needed for the production and isolation of these biocatalysts to determine at least the most important biochemical properties as enzymatic activity, substrate specificity, or enantioselectivity. Apart from Gram-positive bacteria like *Bacillus subtilis*, the genus *Pseudomonas* has emerged as a reasonable alternative to the well-known overexpression systems operating in *E. coli*. *P. aeruginosa* is known for a long time as an opportunistic pathogen which possesses and uses several different pathways to secrete a large number of extracellular enzymes which can also act as virulence factors in bacterial infections. Obviously, the rule 'good pathogens are good secretors' applies here and *P. aeruginosa* has therefore been the object of intensive studies to elucidate different protein secretion pathways at a molecular resolution. These studies have revealed a number of proteins which either themselves contribute to build up a secretion machinery or they are involved in catalyzing the folding or the secretion process. In the near future, these results will be transferred to construct *Pseudomonas* production strains from non-pathogenic species

other than *P. aeruginosa*. Undoubtedly, *Pseudomonas* bacteria will belong to the tool box of novel and efficient biocatalyst overexpression systems.

REFERENCES

1. Ahn, J.H., Pan, J.G., and Rhee, J.S., 1999, Identification of the tliDEF ABC transporter specific for lipase in *Pseudomonas fluorescens* SIK W1. *J. Bacteriol.*, 181:1847–1852.
2. Ahn, J.H., Pan, J.G., and Rhee, J.S., 2001, Homologous expression of the lipase and ABC transporter gene cluster, tliDEFA, enhances lipase secretion in *Pseudomonas* spp. *Appl. Environ. Microbiol.*, 67:5506–5511.
3. Akatsuka, H., Kawai, E., Omori, K., Komatsubara, S., Shibatani, T., and Tosa, T., 1994, The lipA gene of *Serratia marcescens* which encodes an extracellular lipase having no N-terminal signal peptide. *J. Bacteriol.*, 176(7):1949–1956.
4. Andersen, C., Koronakis, E., Bokma, E., Eswaran, J., Humphreys, D., Hughes, C., and Koronakis, V., 2002, Transition to the open state of the TolC periplasmic tunnel entrance. *Proc. Natl. Acad. Sci. USA*, 99:11103–11108.
5. Arnold, F.H., 2001, Combinatorial and computational challenges for biocatalyst design. *Nature*, 409:253–257.
6. Arpigny, J.L. and Jaeger, K.E., 1999, Bacterial lipolytic enzymes: Classification and properties. *Biochem. J.*, 343:177–183.
7. Baneyx, F., 1999, Recombinant protein expression in *Escherichia coli*. *Curr. Opin. Biotechnol.*, 10:411–421.
8. Barrett, C.M., Ray, N., Thomas, J.D., Robinson, C., and Bolhuis, A., 2003, Quantitative export of a reporter protein, GFP, by the twin-arginine translocation pathway in *Escherichia coli*. *Biochem. Biophys. Res. Commun.*, 304:279–284.
9. Berks, B.C., Sargent, F., De Leeuw, E., Hinsley, A.P., Stanley, N.R., Jack, R.L., Buchanan, G., and Palmer, T., 2000, A novel protein transport system involved in the biogenesis of bacterial electron transfer chains. *Biochim. Biophys. Acta*, 1459:325–330.
10. Berks, B.C., Sargent, F., and Palmer, T., 2000, The Tat protein export pathway. *Mol. Microbiol.*, 35:260–274.
11. Binet, R., Letoffe, S., Ghigo, J.M., Delepelaire, P., and Wandersman, C., 1997, Protein secretion by Gram-negative bacterial ABC exporters. *Gene*, 192:7–11.
12. Braun, P., Gerritse, G., van Dijl, J.M., and Quax, W.J., 1999, Improving protein secretion by engineering components of the bacterial translocation machinery. *Curr. Opin. Biotechnol.*, 10:376–381.
13. Braun, P., Ockhuijsen, C., Eppens, E., Koster, M., Bitter, W., and Tommassen, J., 2001, Maturation of *Pseudomonas aeruginosa* elastase. Formation of the disulfide bonds. *J. Biol. Chem.*, 276:26030–26035.
14. Brunschwig, E. and Darzins, A., 1992, A two-component T7 system for the overexpression of genes in *Pseudomonas aeruginosa*. *Gene*, 111:35–41.
15. Cao, T.B. and Saier, M.H. Jr, 2003, The general protein secretory pathway: Phylogenetic analyses leading to evolutionary conclusions. *Biochim. Biophys. Acta.*, 1609:115–125.
16. Chabeaud, P., de Groot, A., Bitter, W., Tommassen, J., Heulin, T., and Achouak, W., 2001, Phase-variable expression of an operon encoding extracellular alkaline protease, a serine protease homolog, and lipase in *Pseudomonas brassicacearum*. *J. Bacteriol.*, 183:2117–2120.
17. Chihara-Siomi, M., Yoshikawa, K., Oshima-Hirayama, N., Yamamoto, K., Sogabe, Y., Nakatani, T., Nishioka, T., and Oda, J., 1992, Purification, molecular cloning, and expression of lipase from *Pseudomonas aeruginosa*. *Arch. Biochem. Biophys.*, 296:505–513.

18. Collet, J.F. and Bardwell, J.C., 2002, Oxidative protein folding in bacteria. *Mol. Microbiol.*, 44:1–8.
19. Cornelis, P., 2000, Expressing genes in different *Escherichia coli* compartments. *Curr. Opin. Biotechnol.*, 11:450–454.
20. Duong, F., Soscia, C., Lazdunski, A., and Murgier, M., 1994, The *Pseudomonas fluorescens* lipase has a C-terminal secretion signal and is secreted by a three-component bacterial ABC-exporter system. *Mol. Microbiol.*, 11:1117–1126.
21. El Khattabi, M., Ockhuijsen, C., Bitter, W., Jaeger, K.E., and Tommassen, J., 1999, Specificity of the lipase-specific foldases of gram-negative bacteria and the role of the membrane anchor. *Mol. Gen. Genet.*, 261:770–776.
22. El Khattabi, M., Van Gelder, P., Bitter, W., and Tommassen, J., 2000, Role of the lipase-specific foldase of *Burkholderia glumae* as a steric chaperone. *J. Biol. Chem.*, 275:26885–26891.
23. El Khattabi, M., Rosenau, F., Bitter, W., Jaeger, K.E., and Tommassen, J., 2003, Folding mediated by the lipase-specific foldases is a bottleneck in the production of *Burkholderia glumae* and *Pseudomonas aeruginosa* lipases. *Appl. Env. Microbiol.*, in press.
24. Filloux, A., Michel, G., and Bally, M., 1998, GSP-dependent protein secretion in gram-negative bacteria: The Xcp system of *Pseudomonas aeruginosa*. *FEMS Microbiol. Rev.*, 22:177–198.
25. Frenken, L.G.J., Bos, J.W., Visser, C., Müller, W., Tommassen, J., and Verrips, C.T., 1993, An accessory gene, lipB, required for the production of active *Pseudomonas glumae* lipase. *Mol. Microbiol.*, 9:579–589.
26. Frenken, L.G.J., de Groot, A., Tommassen, J., and Verrips, C.T., 1993, Role of lipB gene product in the folding of the secreted lipase of *Pseudomonas glumae*. *Mol. Microbiol.*, 9:591–599.
27. Gerritse, G., Ure, R., Bizoullier, F., and Quax, W.J., 1998, The phenotype enhancement method identifies the Xcp outer membrane secretion machinery from *Pseudomonas alcaligenes* as a bottleneck for lipase production. *J. Biotechnol.*, 64:23–38.
28. Gøthel, S.F. and Marahiel, M.A., 1999, Peptidyl-prolyl cis-trans isomerases, a superfamily of ubiquitous folding catalysts. *Cell. Mol. Life Sci.*, 55:423–436.
29. Hoang, T.T., Kutchma, A.J., Becher, A., and Schweizer, H.P., 2000, Integration-proficient plasmids for *Pseudomonas aeruginosa*: Site-specific integration and use for engineering of reporter and expression strains. *Plasmid*, 43:59–72.
30. Ihara, F., Okamoto, I., Akao, K., Nihira, T., and Yamada, Y., 1995, Lipase modulator protein (LimL) of *Pseudomonas* sp. strain 109. *J. Bacteriol.*, 177:1254–1258.
31. Iizumi, T., Nakamura, K., Shimada, Y., Sugihara, A., Tominaga, Y., and Fugase, T., 1991, Cloning, nucleotide sequencing, and expression in *Escherichia coli* of a lipase and its activator genes from *Pseudomonas* sp. KWI-56. *Agric. Biol. Chem.*, 55:2349–2357.
32. Jaeger, K.E., Ransac, S., Dijkstra, B.W., Colson, C., van Heuvel, M., and Misset, O., 1994, Bacterial lipases. *FEMS Microbiol. Rev.*, 15:29–63.
33. Jaeger, K.E., Liebeton, K., Zonta, A., Schimossek, K., and Reetz, M.T., 1996, Biotechnological application of *Pseudomonas aeruginosa* lipase: Efficient kinetic resolution of amine and alcohols. *Appl. Microbiol. Biotechnol.*, 46:99–105.
34. Jaeger, K.E., Schneidinger, B., Rosenau, F., Werner, M., Lang, D., Dijkstra, B.W., Zonta, A., and Reetz, M.T., 1997, Bacterial lipases for biotechnological applications. *J. Mol. Catal. B: Enzymatic*, 3:3–12.
35. Jaeger, K.E. and Reetz, M.T., 1998, Microbial lipases form versatile tools for biotechnology. *Trends Biotechnol.*, 16:396–403.
36. Jaeger, K.E., Dijkstra, B.W., and Reetz, M.T., 1999, Bacterial biocatalysts: Molecular biology, three-dimensional structures, and biotechnological applications of lipases. *Annu. Rev. Microbiol.*, 53:315–351.
37. Jaeger, K.E. and Reetz, M.T., 2000, Directed evolution of enantioselective enzymes for organic chemistry. *Curr. Opin. Chem. Biol.*, 4:68–73.

38. Jaeger, K.E., Eggert, T., Eipper, A., and Reetz, M.T., 2001, Directed evolution and the creation of enantioselective biocatalysts. *Appl. Microbiol. Biotechnol.*, 55:519–30.
39. Jaeger, K.E., Liebeton, K., Eggert, T., Nardini, M., Dijkstra, B.W., Zha, D., Wilensek, S., Zonta, A., Hermes, M., Eipper, A., and Reetz, M.T., 2001, *Directed Evolution of an Enantioselective Lipase* (abstract). Abstracts of the 6th Annual World Congress on Enzyme Technologies, 26–28 February, San Diego, USA.
40. Jaeger, K.E. and Eggert, T., 2002, Lipases for biotechnology. *Curr. Opin. Biotechnol.*, 13:390–397.
41. Jorgensen, S., Skov, K.W., and Diderichsen, B., 1991, Cloning, sequence, and expression of a lipase gene from *Pseudomonas cepacia*: Lipase production in heterologous hosts requires two *Pseudomonas* genes. *J. Bacteriol.*, 173:559–567.
42. Kawai, E., Idei, A., Kumura, H., Shimazaki, K., Akatsuka, H., and Omori, K., 1999, The ABC-exporter genes involved in the lipase secretion are clustered with the genes for lipase, alkaline protease, and serine protease homologues in *Pseudomonas fluorescens* no. 33. *Biochim. Biophys. Acta*, 1446:377–382.
43. Kim, E.K., Jang, W.H., Ko, J.H., Kang, J.S., Noh, M.J., and Yoo, O.J., 2001, Lipase and its modulator from *Pseudomonas* sp. strain KFCC 10818: Proline-to-glutamine substitution at position 112 induces formation of enzymatically active lipase in the absence of the modulator. *J. Bacteriol.*, 183:5937–5941.
44. Kok, R.G., van Thor, J.J., Nutgeren-Roodzant, I., Vosman, B., and Hellingwerf, K.J., 1995, Characterization of lipase-deficient mutants of *Acinetobacter calcoaceticus* BD413: Identification of a periplasmic lipase chaperone essential for the production of extracellular lipase. *J. Bacteriol.*, 177:3295–3307.
45. Kovach, M.E., Phillips, R.W., Elzer, P.H., Roop, R.M. II, and Peterson, K.M., 1994, pBBR1MCS: A broad-host-range cloning vector. *Biotechniques*, 16:800–802.
46. Liebeton, K., Zonta, A., Schimossek, K., Nardini, M., Lang, D., Dijkstra, B.W., Reetz, M.T., and Jaeger, K.E., 2000, Directed evolution of an enantioselective lipase. *Chem. Biol.*, 7:709–718.
47. Liebeton, K., Zacharias, A., and Jaeger, K.E., 2001, Disulfide bond in *Pseudomonas aeruginosa* lipase stabilizes the structure but is not required for interaction with its foldase. *J. Bacteriol.*, 183:597–603.
48. Liese, A., Seelbach, K., and Wandrey, C., 2000, *Industrial Biotransformations*. Wiley-VCH, Weinheim.
49. Lorenz, P., Liebeton, K., Niehaus, F., and Eck, J., 2002, Screening for novel enzymes for biocatalytic processes: Accessing the metagenome as a resource of novel functional sequence space. *Curr. Opin. Biotechnol.*, 13:572–577.
50. Ma, Q., Zhai, Y., Schneider, J.C., Ramseier, T.M., and Saier, M.H., 2003, Protein secretion systems of *Pseudomonas aeruginosa* and *P. fluorescens*. *Biochim. Biophys. Acta*, 1611:223–233.
51. Martinez, A., Ostrovsky, P., and Nunn, D.N., 1999, LipC, a second lipase of *Pseudomonas aeruginosa*, is LipB and Xcp dependent and is transcriptionally regulated by pilus biogenesis components. *Mol. Microbiol.*, 34:317–326.
52. Nardini, M., Lang, D.A., Liebeton, K., Jaeger, K.E., and Dijkstra, B.W., 2000, Crystal structure of *Pseudomonas aeruginosa* lipase in the open conformation. The prototype for family I.1 of bacterial lipases. *J. Biol. Chem.*, 275:31219–31225.
53. Nishijyo, T., Haas, D., and Itoh, Y., 2001, The CbrA-CbrB two-component regulatory system controls the utilization of multiple carbon and nitrogen sources in *Pseudomonas aeruginosa*. *Mol. Microbiol.*, 40:917–931.
54. Ochsner, U.A., Snyder, A., Vasil, A.I., and Vasil, M.L., 2002, Effects of the twin-arginine translocase on secretion of virulence factors, stress response, and pathogenesis. *Proc. Natl. Acad. Sci. USA*, 99:8312–8317.

55. Ogierman, M.A., Fallarino, A., Riess, T., Williams, S.G., Attridge, S.R., and Manning, P., 1997, Characterization of the *Vibrio cholerae* El Tor lipase operon lipAB and a protease gene downstream of the hyl region. *Microbiology*, 140:931–943.
56. Oshima-Hirayama, N., Yoshikawa, K., Nishioka, T., and Oda, J., 1993, Lipase from *Pseudomonas aeruginosa*: Production in *Escherichia coli* and activation in vitro with a protein from the downstream gene. *Eur. J. Biochem.*, 215:239–246.
57. Powell, K.A., Ramer, S.W., Del Cardayre, S.B., Stemmer, W.P., Tobin, M.B., Longchamp, P.F., and Huisman, G.W., 2001, Directed evolution and biocatalysis. *Angew. Chem. Int. Ed. Engl.*, 40:3948–3959.
58. Pugsley, A.P., 1992, Superfamilies of bacterial transport systems with nucleotide binding components. *Symp. Soc. Gen. Microbiol.*, 47:223–248.
59. Raivio, T.L. and Silhavy, T.J., 2001, Periplasmic stress and ECF sigma factors. *Annu. Rev. Microbiol.*, 55:591–624.
60. Reetz, M.T., Zonta, A., Schimossek, K., Liebeton, K., and Jaeger, K.E., 1997, Creation of enantioselective biocatalysts for organic chemistry. *Angew. Chem. Int. Ed.*, 36:2830–2832.
61. Reetz, M.T. and Jaeger, K.E., 1999, Superior biocatalysts by directed evolution. *Top. Curr. Chem.*, 200:32–57.
62. Reetz, M.T. and Jaeger, K.-E., 2000, Enantioselective enzymes for organic synthesis created by directed evolution. *Chem. Eur. J.*, 6:407–412.
63. Reetz, M.T., Wilensek, S., Zha, D., and Jaeger, K.E., 2001, Directed evolution of an enantioselective enzyme through combinatorial multiple-cassette mutagenesis. *Angew. Chem. Int. Ed. Engl.*, 40:3589–3591.
64. Reetz, M.T., 2002, Lipases as practical biocatalysts. *Curr. Opin. Chem. Biol.*, 6:145–150.
65. Reetz, M.T. and Jaeger, K.E., 2002, Directed evolution as a means to create enantioselective enzymes for use in organic chemistry. In S. Brakmann and K. Johnson (eds), *Directed Molecular Evolution of Proteins*, pp. 245–279. Wiley-VCH, Weinheim.
66. Rosenau, F. and Jaeger, K.E., 2000, Bacterial lipases from *Pseudomonas*: Regulation of gene expression and mechanisms of secretion. *Biochimie*, 82:1023–1032.
67. Rosenau, F. and Jaeger, K.E., 2003, Overexpression and secretion of biocatalysts in *Pseudomonas*. In A. Svendsen (ed.), *Enzyme Functionality: Design, Engineering and Screening*, pp. 622–633. Marcel Dekker Publishers, New York, USA.
68. Sandkvist, M., 2001, Biology of type II secretion. *Mol. Microbiol.*, 40:271–283.
69. Schiene, C. and Fischer, G., 2000, Enzymes that catalyse the restructuring of proteins. *Curr. Opin. Struct. Biol.*, 10:40–45.
70. Schmid, R.D. and Verger, R., 1998, Lipases: Interfacial enzymes with attractive applications. *Angew. Chem. Int. Ed. Engl.*, 37:1608–1633.
71. Schmid, A., Dordick, J.S., Hauer, B., Kiener, A., Wubbolts, M., and Witholt, B., 2001, Industrial biocatalysis today and tomorrow. *Nature*, 409:258–268.
72. Schoemaker, H.E., Mink, D., and Wubbolts, M.G., 2003, Dispelling the myths—biocatalysis in industrial synthesis. *Science*, 299:1694–1697.
73. Schweizer, H.P., 2001, Vectors to express foreign genes and techniques to monitor gene expression in *Pseudomonads*. *Curr. Opin. Biotechnol.*, 12:439–445.
74. Sullivan, E.R., Leahy, J.G., and Colwell, R.R., 1999, Cloning and sequence analysis of the lipase and lipase chaperone-encoding genes from *Acinetobacter calcoaceticus* RAG-1, and redefinition of a proteobacterial lipase family and an analogous lipase chaperone family. *Gene*, 230:277–286.
75. Upton, C. and Buckley, J.T., 1995, A new family of lipolytic enzymes? *Trends Biochem. Sci.*, 20:178–179.
76. Urban, A., Leipelt, M., Eggert, T., and Jaeger, K.E., 2001, DsbA and DsbC affect extracellular enzyme formation in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 183:587–596.

77. Voulhoux, R., Ball, G., Ize, B., Vasil, M.L., Lazdunski, A., Wu, L.F., and Filloux, A., 2001, Involvement of the twin-arginine translocation system in protein secretion via the type II pathway. *EMBO J.*, 20:6735–6741.
78. Watson, A.A., Alm, R.A., and Mattick, J.S., 1996, Construction of improved vectors for protein production in *Pseudomonas aeruginosa*. *Gene*, 172:163–164.
79. Wilhelm, S., Tommassen, J., and Jaeger, K.E., 1999, A novel lipolytic enzyme located in the outer membrane of *Pseudomonas aeruginosa*. *J. Bacteriol.*, 181:6977–6986.
80. Wohlfahrt, S., Hoesche, C., Strunk, C., and Winkler, U.K., 1992, Molecular genetics of the extracellular lipase of *Pseudomonas aeruginosa* PAO1. *J. Gen. Microbiol.*, 138:1325–1335.
81. Yen, M.R., Peabody, C.R., Partovi, S.M., Zhai, Y., Tseng, Y.H., and Saier, M.H., 2002, Protein-translocating outer membrane porins of Gram-negative bacteria. *Biochim. Biophys. Acta*, 1562:6–31.
82. Zha, D., Wilensek, S., Hermes, M., Jaeger, K.E., and Reetz, M.T., 2001, Complete reversal of enantioselectivity of an enzyme-catalyzed reaction by directed evolution. *Chem. Commun.* (Cambridge), 2664–2665.

DEGRADATION OF CHLOROAROMATICS BY PSEUDOMONA(D)S

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1. INTRODUCTION

Chlorinated hydrocarbons comprise a large spectrum of compounds that are of enormous industrial and economic importance because of their applications and global outputs. Apart from the common feature of having one or more covalent bound chlorine atoms, these compounds show a complex diversity of behavior that is primarily characterized by their aliphatic or aromatic character and the presence of other functional groups. Nevertheless, the introduction of chlorine atom(s) into a hydrocarbon significantly influences its physicochemical and biochemical properties and the tendency for bioaccumulation and environmental persistence. Acting in combination with possible (eco)toxicological effects, these properties have pushed the chlorochemistry into the focus of considerable debate and governmental regulatory action.

Pseudomonas strains are well known for their widespread occurrence in natural habitats and their ability to utilize a great variety of organic compounds, including haloaromatics. In classical taxonomy, the pseudomonads constitute a group of Gram-negative, rod-shaped, non-spore-forming, polar-flagellated bacteria whose G+C content is higher than 50%. Molecular studies showed that the former group of pseudomonads does not form a homogeneous entity but consist of phylogenetically unrelated genera. On the basis of hybridization experiments, a clear circumscription of five main rRNA similarity clusters was apparent²⁶⁴. In addition, some groups were found to be closer to species of other genera than to those of other *Pseudomonas* groups. Many of the organisms originally described as species of the genus *Pseudomonas* have, in the meantime, been reclassified to other genera such as *Burkholderia*⁴⁰¹, *Ralstonia*⁴⁰² (rRNA group II, *Burkholderia* group of the β -Proteobacteria), *Comamonas*³⁵⁸, *Delftia*³⁸³, *Acidovorax*³⁸⁸ (rRNA group III, Comamonadaceae, β -Proteobacteria), *Brevundimonas*³³⁵ (rRNA group IV, Caulobacter group of the α -Proteobacteria) or *Xanthomonas/Stenotrophomonas*²⁶³ (rRNA group V, *Xanthomonas* group of the γ -Proteobacteria). Only species of rRNA group I (belonging to the γ -Proteobacteria) are currently classified as *Pseudomonas* (*sensu stricto*)²³², comprising the *P. syringae*, *P. chlororaphis*, *P. fluorescens*, *P. putida*, *P. stutzeri*, *P. aeruginosa* and *P. pertucinogena* groups⁴. For recent overviews on the taxonomy of *Pseudomonas* (see Chapter 1 in Volume 1 by Palleroni and Moore or refs [4], [162], [232]).

This chapter deals primarily with the capabilities of *Pseudomonas* (*sensu stricto*). However, several reports dealing with the degradation of chloroaromatic compounds and enzyme mechanisms only provide tentative identifications, such that designation of isolates as *Pseudomonas* has to be taken with care in various cases.

2. THE CHLOROCATECHOL PATHWAY, A CENTRAL CATABOLIC ROUTE

2.1. The Chlorocatechol Pathway of *Pseudomonas* sp. Strain B13

The first observations of microorganisms being capable of degrading chloroaromatics were made in the 1960s by the groups of Alexander and Evans, and first indications of the metabolic fate of chlorophenoxyacetates via chlorocatechols as central intermediates were given^{28, 84, 103, 362}. However, the basic knowledge on a dominant degradation pathway for chloroaromatics was accumulated using one of the first *Pseudomonas* strains capable of degrading chloroaromatics, *Pseudomonas* sp. strain B13⁶⁷. Strain B13 is capable of mineralizing 3-chlorobenzoate via 3-chloro- and 4-chlorocatechol as central

intermediates. Degradation is initiated by a chromosomal encoded benzoate dioxygenase forming 3-chloro- and 5-chlorodihydrodihydroxybenzoate in a 2:1 ratio²⁹³, followed by dehydrogenation resulting in 3-chloro- and 4-chlorocatechol²⁹⁴. Chlorocatechols are further degraded by a chlorocatechol *ortho*-cleavage pathway^{68, 69, 156, 318, 319}, which differs from the 3-oxoadipate pathway for catechol degradation¹²² in various aspects (Figure 1). Degradation is initiated by a broad specificity chlorocatechol 1,2-dioxygenase with consumption of molecular oxygen to produce the corresponding chloro-*cis*, *cis*-muconates^{36, 68, 69, 319, 362}. The elimination of the chlorine substituent was assumed to occur spontaneously after 2-chloro- and 3-chloro-*cis*, *cis*-muconate have been converted by chloromuconate cycloisomerases to 5-chloro- and 4-chloromuconolactone, respectively³¹⁸. The formed dienelactones (*cis*-dienelactone from 3-chloro- and *trans*-dienelactone from 2-chloro-*cis*, *cis*-muconate) are converted by a dienelactone hydrolase into maleylacetate³¹⁸, and a maleylacetate reductase catalyzes the reduction of the double bond to form 3-oxoadipate, the common metabolite of the 3-oxoadipate and the chlorocatechol *ortho*-cleavage pathway¹⁵⁶. It was thus clear, that a special set of enzymes is responsible for degradation of chlorocatechol intermediates. The genetic background for chlorocatechol degradation in *Pseudomonas* was initially elucidated using an independently isolated 3-chlorobenzoate degrading *Pseudomonas putida* strain AC858^{47, 48}, and it was observed that the genes encoding enzymes of chlorocatechol pathway were located on a plasmid.

Mating methods mimicking natural gene transfer were used to transfer genes for chlorocatechol degradation from strain B13 to various recipients^{291, 295}. It had been suspected that the genes were transferred by means of a conjugative plasmid, pB13⁴⁶, however, independent isolation remained unsuccessful³⁸². Ravatn *et al.*²⁸⁸ found that the transferred element was not a classical plasmid. In fact, when strain B13 was mated with *P. putida* F1, the transferred DNA, named the *clc* element, behaved rather strangely and formed large, unstable duplications that were integrated into the chromosome at two locations²⁸⁷. The 105 kb element containing the genes for chlorocatechol degradation (among others) was also discovered in the chromosome of strain B13, in two separate locations²⁸⁸. It integrates into the 3'-end of a target glycine tRNA gene and obviously employs a phage-type integrase to achieve site-specific integrations^{288, 374}. With a certain frequency the element excises, resulting in a circular intermediate. In contrast to many pathogenicity islands, the *clc* element (or *clc* genomic island) is self-transmissible to other recipient bacteria³⁴⁷.

2.2. Chlorocatechol Genes in *Pseudomonas*(d)s

Chlorocatechol genes were not only observed in the above-mentioned *Pseudomonas* strains, but also in *P. aeruginosa* JB2¹³², *Pseudomonas* sp. strain

P51³⁷³, and various other proteobacteria like *R. eutropha* JMP134^{64, 65}, *R. eutropha* NH9²⁵⁴ or *Burkholderia* sp. strain NK8²⁰¹. Usually, these genes are localized on catabolic plasmids⁴⁷, often broad host range IncP-1 plasmids³⁶⁶, such as pJP4 of strain JMP134⁶⁵. Just recently, Müller *et al.*²³⁶ showed that also genomic islands containing chlorocatechol catabolic genes are not unique to strain B13, and a genomic island very similar to the *clc* genomic island was identified in *Ralstonia* sp. strain JS705 isolated from a chlorobenzene-contaminated environment. The fact that chlorocatechol genes are localized on mobile genetic elements indicated that they can be easily transferred, and thus spread in the environment.

The genes encoding the chlorocatechol pathway in all the above organisms form clusters and the structures of the corresponding operons are nearly identical, in spite of the geographically distinct origins of the bacteria or the difference in their phylogenetic position (Figure 1). The structural gene encoding chlorocatechol dioxygenase is followed by the structural gene encoding chloromuconate cycloisomerase. The major difference between the different clusters is that one group (e.g., the gene clusters of *P. putida* AC858⁴⁸, *P. aeruginosa* JB2¹³², *Pseudomonas* sp. strain P51³⁷³, *P. chlororaphis* RW71²⁸¹, *R. eutropha* NH9²⁵⁴) contains an ORF of unknown function following the chloromuconate cycloisomerase gene whereas such an ORF is absent in the other (e.g., strains JMP134 and NK8^{152, 201}). Two structural genes encoding dienelactone hydrolase and maleylacetate reductase complete the catabolic clusters^{89, 132, 154, 272, 373}. Regulatory genes usually precede the structural genes and are orientated in the opposite direction. The gene products of the regulatory genes, which are members of the LysR family, act as positive regulators⁴⁹. 2-Chloro-*cis,cis*-muconate was demonstrated to be the inducer of the *clc* operon^{220, 253}. But not only the structure of the operons of the chlorocatechol genes is similar. Isofunctional enzymes of the chlorocatechol pathway were shown to be homologous and more similar to each other than to the corresponding enzymes of catechol pathways, indicating a common origin of the proteobacterial chlorocatechol pathways³¹⁵. In contrast, the enzymes of the Gram-positive organism *Rhodococcus opacus* 1CP turned out to have unusual biochemical properties and sequences with relatively little similarity to proteobacterial chlorocatechol gene sequences, thus indicating an independent origin^{83, 229, 342}.

2.3. Differences in Chlorocatechol Catabolic Enzymes

However, despite the high similarity in chlorocatechol pathway catabolic genes, significant differences were also observed. Strains like B13 have been isolated for growth on 3-chlorobenzoate. The chlorocatechol gene products were, however, capable of dealing with higher chlorinated catechols such as 3,5-dichloro- or 3,6-dichlorocatechol, whereas 3,4-dichlorocatechol was only a poor substrate^{68, 274}. In contrast, the enzymes of *Pseudomonas* sp. strain P51 (isolated with 1,2,4-trichlorobenzene)³⁷³ or *P. chlororaphis* RW71 (isolated with

1,2,3,4-tetrachlorobenzene)²⁸¹ were capable of transforming 3,4-dichlorocatechol, and strain RW71 was even capable of transforming tetrachlorocatechol with a reasonable activity. Differences were also observed in chloromuconate turnover, with the cycloisomerase of JMP134 being poorly active against 2-chloromuconate^{184, 278, 378}. However, all proteobacterial chloromuconate cycloisomerases obviously share common features, which discriminate them from the *Rhodococcus* enzymes. They catalyze a specific cycloisomerization of 2-chloromuconates, which can occur either between the C1 and C4 or the C3 and C6 carbon atoms to form preferentially 5-chloromuconolactone. 5-Chloromuconolactone is a substrate for proteobacterial chloromuconate cycloisomerases and is dehalogenated to form *trans*-dienelactone³⁷⁹. Thus, proteobacterial chloromuconate cycloisomerases are specific dehalogenases. The formation of *trans*-dienelactone is due to the fact that after cycloisomerization the lactone ring has to rotate in the catalytic center to achieve proton abstraction and thus dehalogenation³¹³. In contrast, chloride from the 3-position of chloromuconate seems to be directly eliminated during cycloisomerization¹⁵⁹. Such mechanisms explain also the differences in stereochemistry of products formed during cycloisomerization of higher chlorinated muconates²⁷⁹. Evidently, for the degradation of various chloroaromatics, it is an important prerequisite that the dienelactone hydrolase involved in the pathway is of relaxed substrate specificity and accepts both *cis*- and *trans*-isomers of dienelactone as substrates³¹⁷. The formed maleylacetates are then transformed by a maleylacetate reductase¹⁵⁶. Maleylacetates with chlorine substituents in 2-position such as 2-chloromaleylacetate (formed from 3,5-dichloro-, and 3,6-dichlorocatechol; Figure 1) or 2,5-dichloromaleylacetate (probably formed from 3,4,6-trichlorocatechol during 1,2,4-trichloro- or 1,2,4,5-tetrachlorobenzene degradation^{19, 373}) are reduced in a first step to give maleylacetate or 3-chloromaleylacetate, respectively. Obviously, enzymatic attack on the C2-carbon results in an intermediate, which spontaneously eliminates chloride. Therefore, two moles of NADH per mole substrate are consumed for the conversion of maleylacetates which contain a chlorine substituent in the 2-position^{45, 156, 157, 380}. No significant differences in substrate specificity and chloride elimination were observed for different maleylacetate reductases involved in the chlorocatechol pathway²³³, and obviously also maleylacetate reductases of the hydroquinone pathway (see Chapter 15, Volume 3) can carry out such dehalogenation^{255, 400}. Probably dehalogenation of 2-chloromaleylacetate is a general capability of maleylacetate reductases and based on the enzyme mechanism. Also chromosomal encoded maleylacetate reductases have been described^{278, 317} (AF130250) and hypothetical maleylacetate reductase genes have been observed in sequenced *Ralstonia* genomes. If those genes are, however, also present on *Pseudomonas* chromosomes remains to be elucidated.

Whereas the degradation of chloroaromatics by pseudomonads has been very well elucidated, only poor information exists on the degradation of

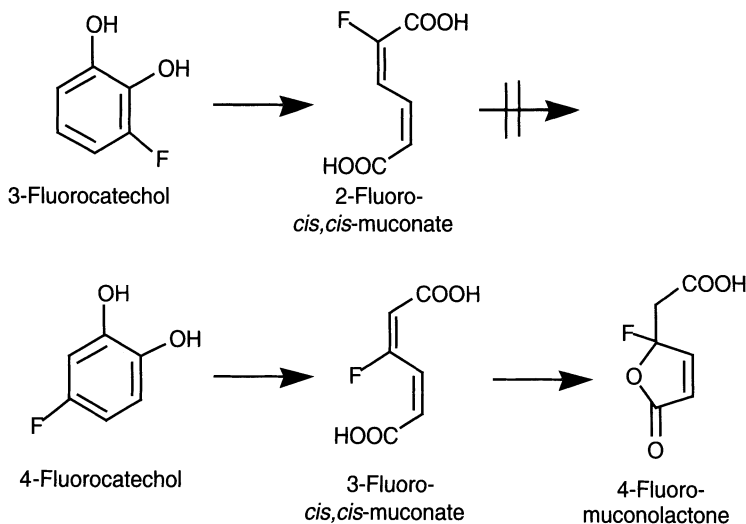


Figure 2. Degradation of fluorocatechols^{77, 316, 318, 325}.

fluoro-, bromo- and iodoaromatic compounds. From literature available it can be deduced that no qualitative difference between the degradability of chloro- and bromocatechols exists and transformation rates of bromo-derivatives are usually slightly lower than those of the respective chlorocatechols^{36, 68}.

Fluorocatechols, however, can create severe problems for proteobacterial chlorocatechol pathway enzymes. Evidently, proteobacterial chlorocatechol pathways are not suited for the degradation of 3-fluorocatechol (Figure 2), and significant cycloisomerization of 2-fluoromuconate is neither achieved by muconate nor chloromuconate cycloisomerases analyzed thus far^{77, 318, 325}. In contrast, degradation of 4-fluorocatechol was reported³²⁵ and the degradation of 4-fluorobenzoate by strain B13 was proposed to proceed via 4-fluorocatechol, 3-fluoromuconate and *cis*-dienelactone. However, Schlömann *et al.*³¹⁶ showed that cycloisomerization of 3-fluoromuconate results in the formation of 4-fluoromuconolactone (Figure 2), which is defluorinated neither by proteobacterial muconate nor chloromuconate cycloisomerases.

3. PERIPHERAL CATABOLIC ROUTES

3.1. Substrate Specificities of Peripheral Enzymes

Pseudomonas sp. strain B13 degrades 3-chlorobenzoate by using chromosomal encoded enzymes in concert with enzymes encoded on the *clc*

element, and various *Pseudomonas* strains have been described capable of degrading 3-chlorobenzoate via a chromosomal encoded benzoate dioxygenase/dehydrogenase system and chlorocatechol genes. Benzoate dioxygenase genes have been localized in *P. putida*, *P. aeruginosa* and *P. fluorescens* genome sequencing projects, and thus seem to be spread among *Pseudomonas* strains. However, in an early study it was observed that in *P. putida*, *P. aeruginosa*, *P. fluorescens*, *P. chlororaphis* and *P. stutzeri* strain collections the capability to degrade benzoate was ubiquitous in *P. aeruginosa*, *P. chlororaphis* and *P. putida*, but only a few *P. stutzeri* and *P. fluorescens* strains harbored that capability³⁴⁸. The substrate specificity of benzoate dioxygenases is usually restricted to benzoate and 3-chloro-/3-methylbenzoate, as shown for chromosomal encoded enzymes from strain B13²⁹³, strain JMP134²⁷³ and from the distantly related enzyme from *Rhodococcus* sp. strain RHA1¹⁶⁶. Thus, by recruitment of a chlorocatechol pathway, such strains can assemble a complete pathway for 3-chlorobenzoate degradation.

In contrast to benzoate dioxygenase, the toluate 1,2-dioxygenase (XylXYZ) of *P. putida* mt-2, whose natural function is the conversion of *m*- and *p*-toluate, transforms also 4-chlorobenzoate²⁹³. 4-Chlorobenzoate is a good substrate because of the structural analogy to *p*-toluate (4-methylbenzoate). These data indicate that electronic effects, which might have been expected from chlorosubstituents, are not responsible for the varying activities found with different chlorobenzoates. Instead, pre-adaptation for growth with different methyl-substituted substrates has eliminated the steric hindrance for a respective chloroanalogous compound. It can be reasoned that pathways evolved for the degradation of naturally occurring methylsubstituted growth substrates can often deal with substrates bearing chlorine substituents, since both substituents are of similar size. This pre-adaptation avoids steric hindrance and slowdown of conversion rate of a chlorinated substrate.

Thus it was reasoned that a combination of a toluate pathway with a chlorocatechol pathway should result in derivative strains capable of mineralizing 4-chlorobenzoate. Actually, strain B13 acquired the ability to utilize novel substrates like 4-chloro- and 3,5-dichlorobenzoate after transfer of the TOL plasmid of *P. putida* mt-2 harboring the toluate dioxygenase genes²⁹⁵. Similarly, transfer of the *clc* element into strain mt-2 resulted in 4-chlorobenzoate degrading derivatives²⁹². However, a simple combination of pathway segments did not directly allow transconjugants to grow on 4-chlorobenzoate, but 4-chlorocatechol formed from 4-chlorobenzoate was dominantly channeled into the catechol *meta*-cleavage pathway (see Section 4.2), the genes of which are localized in the same transcriptional unit on the TOL plasmid as the toluate dioxygenase genes. In 4-chlorobenzoate mineralizing hybrid strains misrouting was avoided either by inactivating the gene of the catechol 2,3-dioxygenase, *xylE*, by an insertion^{146, 389} or a point mutation (P. A. Williams,

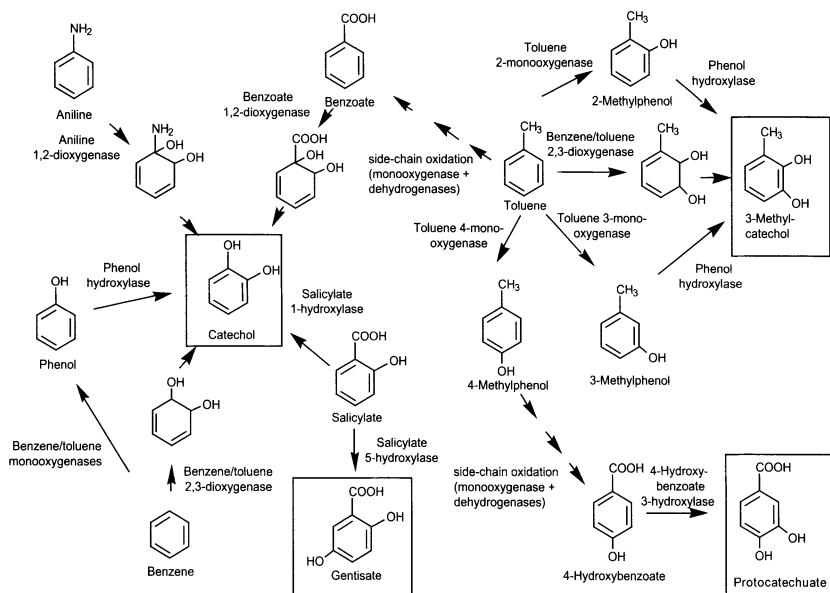


Figure 3. Peripheral funneling pathways leading to the formation of central diphenolic intermediates catechol, methylcatechol, protocatechuate or gentisate (shown boxed).

personal communication). By mimicking natural gene transfer in the laboratory the judicious combination of peripheral (Figure 3) and central chlorocatechol pathway segments (Figure 1) in a suitable host organism has provided complete hybrid pathways for various mono- and disubstituted chloroaromatics²⁷⁵ such as chlorinated benzenes²⁵⁸, phenols³²⁸, salicylates³⁰³, anilines¹⁸⁸ and toluenes^{1, 35}.

In the meantime, information on the substrate specificities, genetic organization and spread of such peripheric enzymes, specifically in *Pseudomonas* and other proteobacteria has accumulated. A broad substrate specificity enzyme analyzed in detail is the toluene dioxygenase of *P. putida* F1. Toluene dioxygenase belongs to the toluene/biphenyl family of Rieske non-heme iron dioxygenases¹⁰⁶. The enzyme system comprises an electron transport chain of ferredoxin reductase and ferredoxin, which channels electrons from NADH onto the catalytically active oxygenase consisting of α - and β -subunits (Figure 4)⁴⁰⁵, with the α -subunit being dominantly responsible for substrate specificity²⁰. Toluene is oxidized to the respective *cis*-dihydrodiol¹⁰⁵, which is subsequently dehydrogenated to 3-methylcatechol (Figure 3). This enzyme system also accepts chlorobenzene, *p*-chloro- or *p*-bromotoluene as substrates, which are oxidized to their dihydrodiols and dehydrogenated to the respective catechols (for a review of toluene dioxygenase catalyzed conversions see ref. [143]).

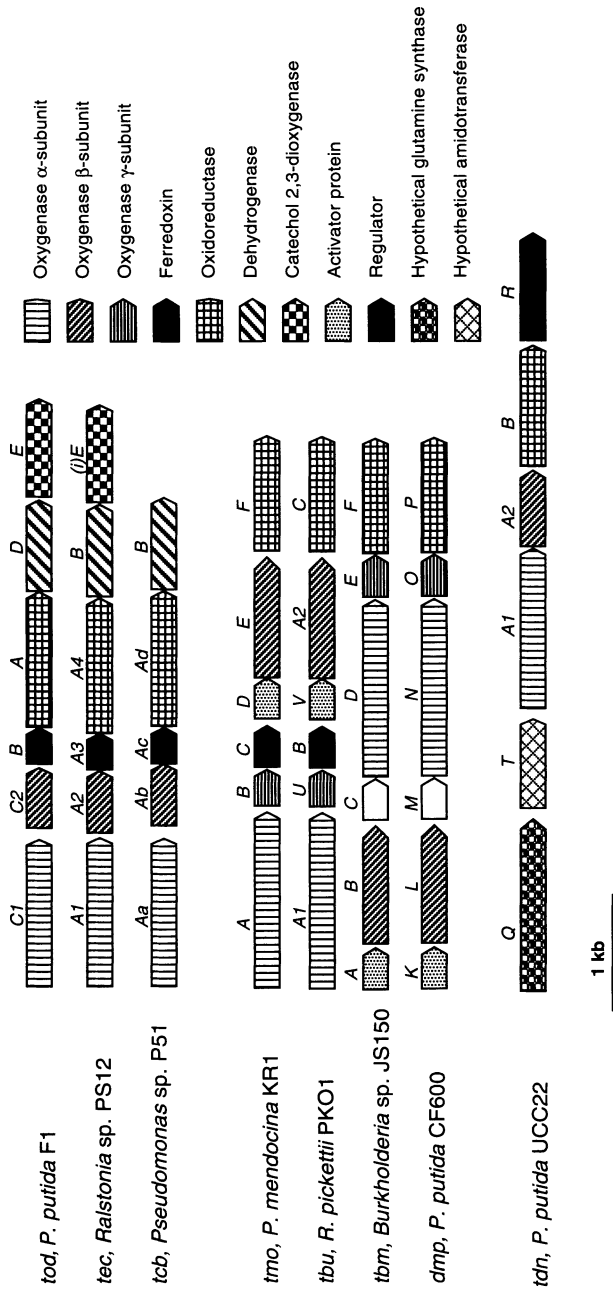


Figure 4. Organization of genes clusters encoding (chloro)benzene dioxigenases^{19, 384, 414} (top part), benzene/toluene monooxygenases or multicomponent phenol hydroxylases^{39, 149, 339, 406, 407} (middle part) and those encoding aniline dioxigenase⁹⁵.

Thus, the introduction of the *clc* element resulted in chlorobenzene degrading derivatives of *P. putida* F1²⁸⁹. Similarly, 1,4-dichlorobenzene degraders can easily be evolved by acquisition of chlorocatechol genes in toluene degrading strains²⁵⁸. Only a few microorganisms have been isolated thus far on the basis of degrading 1,2-dichloro-¹¹⁵, 1,3-dichloro-⁵⁹, or 1,4-dichlorobenzene^{258, 324}. Their chlorobenzene dioxygenases have been indicated to be specifically adapted for transformation of their respective growth substrate. *Pseudomonas* sp. strain P51, isolated on the basis of degrading 1,2,4-trichlorobenzene³⁷⁵ contains a broad-spectrum chlorobenzene dioxygenase capable of transforming all dichlorobenzenes and even 1,2,4-trichlorobenzene. The respective *tcb* genes³⁸⁴ are highly homologous to the respective *tod* genes (Figure 4) involved in toluene degradation by F1 (e.g., 89% identity in amino acid sequence of the respective α -subunits), indicating that small changes in amino acid sequence have a significant influence on substrate specificity. The α -subunit TcbAa also shares more than 96% identity with tetrachlorobenzene dioxygenase of *Ralstonia* sp. PS12, an enzyme that lost the capability to transform benzene¹⁹.

The first enzymes of the chlorobenzene degradation pathway of strain P51, the chlorobenzene dioxygenase and the *cis*-chlorobenzene dihydrodiol dehydrogenase, are encoded on a plasmid-located transposon³⁸⁴ with the chlorocatechol genes located on the same plasmid. High homology suggested that the chlorobenzene dioxygenase and dehydrogenase originated from a toluene or benzene degradation pathway, probably by horizontal gene transfer, to form a functional chlorobenzene degradative pathway in combination with the chlorocatechol genes. A very similar gene organization was observed on the strain PS12 IncP plasmid²¹ and it was shown that different genetic events led to inactivation of the perturbing *meta*-cleavage enzymes in strains P51 and PS12 during the evolution of efficient chlorobenzene degradation pathways. Recently, it could be demonstrated that respective metabolic pathway are formed through the activity of IS elements and *clc* elements under natural conditions²³⁶.

The dioxygenation of toluene is only one of five different aerobic pathways for initializing toluene degradation (Figure 3). The TOL plasmid pWW0 is known to encode not only enzymes for the degradation of *m*- and *p*-toluate (see above) but also the complete set of enzymes necessary for the degradation of *m*- and *p*-xylene via the respective methylbenzoates as intermediates³⁹⁴. The degree of transformation of chlorotoluenes by xylene monooxygenase (XylMA) depends on the position of the chlorine substituent. The substrate analogs 3-chloro- and 4-chlorotoluene are transformed at high rates, while no or only low activity has been found with other chlorotoluenes. Substituents in *ortho* position impaired substrate binding³⁵. Transfer of the TOL plasmid into strain B13 allowed the isolation of chlorotoluene-degrading organisms^{1, 35}.

Toluene monooxygenases that hydroxylate the aromatic nucleus at all three possible positions, producing 2-methyl-, 3-methyl-, or 4-methylphenol (Figure 3) have been described^{256, 338, 385} and include the xylene monooxygenase systems of *P. stutzeri* OX1²³ and *Pseudomonas mendocina* KR1³⁸⁵. These enzymes belong to an evolutionary related family of soluble diiron hydroxylases, including also phenol hydroxylases and methane monooxygenases. The enzyme complexes consist of an electron transport system comprising a reductase (and in some cases a ferredoxin), a catalytic effector protein which contains neither organic cofactors nor metal ions and is assumed to play a role in assembly of an active oxygenase²⁸² and a terminal hydroxylase with a $(\alpha\beta\gamma)_2$ quaternary structure and a diiron center contained in each α -subunit. The monooxygenases were classified according to their α -subunits, which are assumed to be the site of substrate hydroxylation, into four different phylogenetic groups with toluene monooxygenases falling into group 1 and 2¹⁵¹. The enzymes in group 1 comprise primarily phenol and methylphenol monooxygenases like the α -subunit DmpN of the methylphenol degrader *Pseudomonas* sp. strain CF600³³⁹ but also the toluene 2-monooxygenase of *Burkholderia cepacia* G4²⁴⁷ and *Burkholderia* sp. strain JS150¹⁴⁹, and the *tbc1* (phenol hydroxylase) gene cluster of strain JS150. The respective gene clusters usually lack a ferredoxin gene (see Figure 4). As the toluene 2-monooxygenase of strain G4 was capable of further transforming 2-methylphenol formed from toluene into 3-methylcatechol²⁴⁷, this enzyme can be regarded as a phenol hydroxylase (Figure 3). Group 2 comprises enzymes that oxidize nonhydroxylated compounds and the gene cluster usually comprises a ferredoxin component. Some of the enzymes of this subfamily like the *tou* gene products of strain OX1²⁴ have also been shown to oxidize phenol and methylphenols, whereas others do not harbor this capability. In toluene 4-monooxygenase of *P. mendocina* KR1 it was shown that the effector protein is necessary not only for effective coupling, but also for a high regioselectivity²²⁵. Whereas a complete monooxygenase system produced nearly exclusively 4-chlorophenol from chlorobenzene²⁷⁶, absence of the effector protein resulted in a significant formation of 2-chloro- and 3-chlorophenol²²⁵. However, the α -subunit was found to be dominantly responsible for regioselectivity and a G103L mutant was shown to produce nearly equal amounts of 2-chloro-, 3-chloro-, and 4-chlorophenol. The product distribution of the mutant enzyme from toluene was similar to that observed in other naturally occurring members of the toluene/benzene hydroxylase branch of the diiron hydroxylase phylogenetic tree such as *P. aeruginosa* J1104¹⁶⁸ and *P. stutzeri* OX1²³, and it can be proposed that the capability to transform chlorobenzene is spread among those gene products, and actually *tbc2* (toluene 2-monooxygenase) of strain JS150 was shown to transform chlorobenzene into 2-chlorophenol¹⁵¹. Only poor information is available on transformation of chlorophenols formed by

monooxygenation by multicomponent toluene hydroxylases, but Johnson and Olsen¹⁴⁸ indicated the formation of 4-chlorocatechol from chlorobenzene by a plasmid containing toluene 4-monooxygenase genes from strain JS150. The multicomponent phenol hydroxylases, usually localized on plasmids and characterized from various *Pseudomonas* sp. have recently been systematically analyzed, and their phylogenetic relationships correlate with their kinetic properties¹⁰¹. Both, members of the so-called low K_s -group (comprising phenol hydroxylases from *Comamonas testosteroni* R5, *R. eutropha* E2 and toluene 2-monooxygenases of *B. cepacia* G4 and JS100) and the moderate K_s -group (comprising phenol hydroxylases of *Pseudomonas* sp. strain CF600 and *P. putida* BH) have been analyzed for their capability to transform chlorophenols. High chlorophenol dependent oxygen uptake, usually higher than 50% that with phenol was observed by the BH, R5 and E2 derived enzymes, indicating them to be capable of transforming all monochlorophenols³⁶⁰.

The reactions catalyzed by multicomponent phenol hydroxylases (Figure 3) are similar to those catalyzed by flavoprotein monooxygenases¹¹⁸. Both 2,4-dichlorophenol hydroxylases and phenol hydroxylase PheA from *Pseudomonas* sp. strain EST1001 are single component flavoproteins. 2,4-Dichlorophenol hydroxylases are usually involved in 2,4-D degradative pathways, plasmid encoded and linked with further genes involved in 2,4-D degradation^{64, 179, 197}. All three 2,4-dichlorophenol hydroxylases purified so far^{16, 202, 210} do not transform phenol, and activity with 3-chlorophenol was either low, or absent. Despite the fact that PheA shared 46% of sequence identity with TfdB of the 2,4-D degrader strain JMP134, it is of restricted substrate specificity and only transforms phenol and 3-methylphenol²⁵². A phylogenetically unrelated chromosomal encoded flavoprotein phenol hydroxylase, designated TbuD, was described from *Ralstonia pickettii* PKO1¹⁸⁷. The enzyme was shown to exhibit broad substrate specificity and monochlorophenols were transformed to the corresponding catechols¹⁸⁶. Thus, despite the observation that a phenol degrading activity is common in *P. putida* isolates³⁴⁸, the fact that completely different phenol hydroxylase systems are known, prevents us from deducing that a chlorophenol cometabolizing activity is spread in *Pseudomonas* sp. However, for example, *Pseudomonas* sp. B13, obviously recruits a phenol hydroxylase, probably not encoded on the *clc* element, for growth on 4-chlorophenol¹⁷⁶.

Salicylate hydroxylase (Figure 3) is a flavoprotein monooxygenase that catalyzes the conversion of salicylate to catechol. The enzyme was first purified from *P. putida* S1⁴⁰³, later from various other *Pseudomonas* and *Burkholderia*, and cloned and sequenced from various sources^{29, 150, 190, 409}, dominantly *Pseudomonas* sp. Usually, salicylate hydroxylase is included in the naphthalene pathway, and the gene encoding salicylate hydroxylase is followed by genes encoding a *meta*-cleavage pathway. Different so-called NAH

plasmids harboring those genes have been described from *Pseudomonas* strains^{41, 408}, but in contrast, *P. stutzeri* AN10 harbors chromosomal located *nah* genes³⁰². Moreover, variations in gene organization were observed. *P. stutzeri* AN10, besides *nahG* encoding salicylate hydroxylase located in one transcriptional unit with the *meta*-cleavage pathway genes, contains a second gene encoding salicylate hydroxylase, *nahW*, which is situated outside but in close proximity to this transcriptional unit. Both, the *nahG* and *nahW* genes of *P. stutzeri* AN10 are induced and expressed upon incubation with salicylate. Such a gene organization seems to be common to naphthalene degrading *P. stutzeri* strains³⁰. Despite differences in gene organization and partially low homology (*NahW* shares 23–25% amino acid sequence identity with other salicylate hydroxylases), most salicylate hydroxylases described thus far exhibit similar substrate ranges with significant activities against 4-chloro- and 5-chlorosalicylate, and a lower activity against 3-chlorosalicylate^{30, 150, 195, 303}. However, recently a complete new group of three-component salicylate 1-hydroxylases has been described, which consists of a hydroxylase component, a ferredoxin and a ferredoxin reductase²⁷⁷, differing significantly in substrate specificity from previously analyzed single component salicylate 1-hydroxylases²⁷⁷. Given the broad substrate specificity of salicylate 1-hydroxylases it is not astonishing that chlorosalicylate mineralizing *Pseudomonas* strains could easily be obtained by combining a salicylate hydroxylase with a functioning chlorocatechol pathway^{195, 303}. Genes similar to those encoding salicylate hydroxylase (approx. 25–30% amino acid sequence identity) have been localized in the genome of *P. aeruginosa* PAO1 and *P. putida* KT2440, however, KT2440 is reported not to grow on salicylate¹⁴⁷. The analysis of strain collections has shown that only a few *Pseudomonas* strains are capable of mineralizing salicylate, whereas such a capability seems to be frequent in *B. cepacia*³⁴⁸.

In contrast to clear biochemical and genetic evidences for degradation of salicylate/chlorosalicylates, benzenes/chlorobenzenes and benzoates/chlorobenzoates, information on the degradation of aniline/chloroaniline is still scarce. Bacteria from different genera such as *Frateuria*⁵, *Pseudomonas*²¹⁸ or *Acinetobacter*⁹² have been described to degrade aniline via catechol as intermediate (Figure 3), which can then be further metabolized by *ortho*-²³⁸ or *meta*-cleavage pathways³⁰⁷. Such a reaction would suggest the involvement of a dioxygenase system as described for benzoate degradation. However, genetic studies on the plasmid localized genes of *P. putida* UCC22 and *Acinetobacter* sp. strain YAA indicated a more complex mechanism (Figure 4). A dioxygenase system similar to benzoate dioxygenase consisting of α - and β -subunits and a reductase is preceded by genes similar to glutamine synthetases and amidotransferases^{92, 95} indicating their possible involvement in transfer of the amino group. Even though there is no detailed information on substrate specificities of aniline dioxygenases, it can be assumed that in 3-chloroaniline mineralizing

strains the substrate is transformed by aniline dioxygenase, and the formed chlorocatechol degraded via a chlorocatechol *ortho*-cleavage pathway^{135, 188}. Latorre *et al.* analyzed a methylaniline degrading *Pseudomonas* sp. as being capable of cometabolizing chloroanilines and derivatives of *Pseudomonas* sp. strain B13 capable of mineralizing chloroanilines could be obtained after prolonged coculture with this organism, indicating transfer of the aniline dioxygenase system¹⁸⁸. Most isolates reported thus far capable of degrading aniline and chloroanilines belong to the β -subclass of the proteobacteria (dominantly Comamonadaceae^{37, 62}) but also different *Pseudomonas* strains⁶² were reported to harbor such capability.

3.2. Oxygenolytic Dehalogenation

Usually, dioxygenases activating the aromatic ring attack either at two unsubstituted carbon atoms, as shown for benzene/toluene dioxygenase or at an unsubstituted and a carboxylated carbon atom such as benzoate/toluate dioxygenases, giving rise to *cis*-dihydrodiols, which are further transformed by dehydrogenases to give 1,2-diphenols. Some ring activating dioxygenases can bring about dehalogenation of haloaromatic compounds. Benzoate dioxygenase of strain B13 attacks 2-fluorobenzoate dominantly in a 1,6-fashion⁷⁶, giving rise to a dihydrodiol, which is rearomatized to 3-fluorocatechol (Figure 5). As the ring-cleavage product 2-fluoromuconate is no substrate for proteobacterial (chloro)muconate cycloisomerases, strain B13 cannot grow on 2-fluorobenzoate. However, prolonged adaptation resulted in derivatives, which can grow on this substrate. Obviously, spontaneous mutants in the benzoate dioxygenase arose, which dominantly performed a 1,2-dioxygenation⁷⁶, such that one of the *vic*-hydroxyl groups in the *cis*-dihydrodiol is bound to the same carbon as the fluoro-substituent. From such an instable *vic*-dihydrodiol, the fluoro-substituent will be spontaneously eliminated to give catechol (Figure 5). A similar mechanism had been previously suggested for degradation of 2-fluorobenzoate by another pseudomonad^{108, 224}. However, 2-chlorobenzoate cannot be used as a growth substrate by B13-derivatives, indicating that proteobacterial benzoate dioxygenases cannot accommodate such a voluminous substituent in the *ortho*-position.

Since the 1980s, different groups succeeded in isolating bacteria capable of degrading 2-chlorobenzoate, and thus far, only *Pseudomonas* and *Burkholderia* strains were identified to harbor such capability. The isolates could be grouped into those capable of degrading 2-chlorobenzoate (*B. cepacia* INMI-KZ-2⁴¹¹, *B. cepacia* 2CBS⁸⁶, *P. putida* CLB250⁷⁹, *P. aeruginosa* 2-BBZA¹³³ and *Burkholderia* sp. strain TH2³⁵⁴) and those capable of degrading also either 2,4-dichloro- or 2,5-dichlorobenzoate. The last mentioned group comprises thus far only *Pseudomonas* strains (*P. stutzeri* KS25¹⁸⁰, *P. putida* P111¹²⁸, *P. aeruginosa*

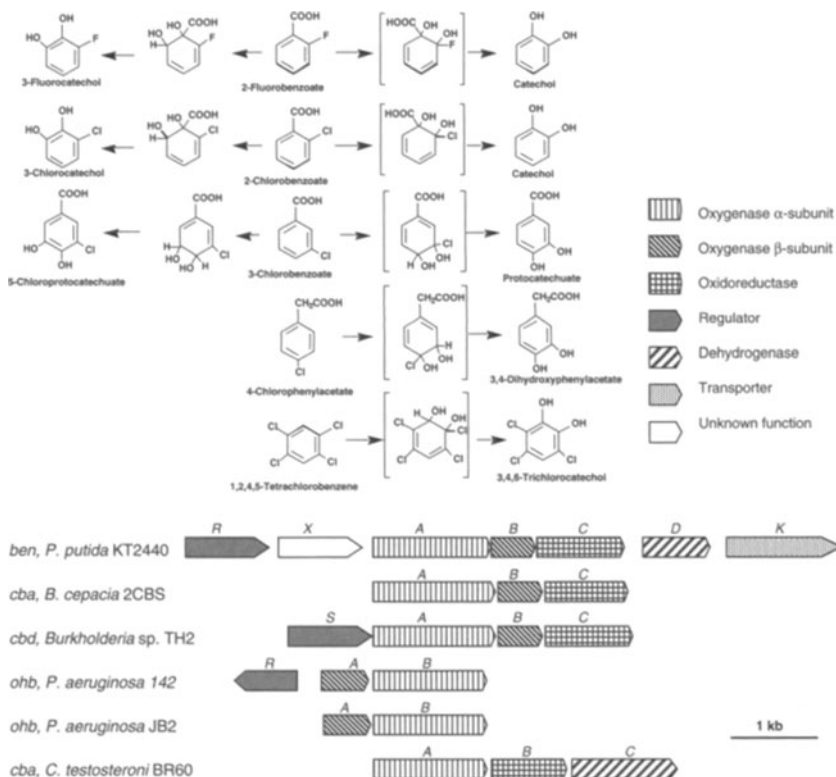


Figure 5. The oxygenolytic dehalogenation of 2-fluoro-⁷⁶, 2-chloro-⁸⁷, and 3-chlorobenzoate²⁴² as well as of 4-chlorophenylacetate²¹³ and of 1,2,4,5-tetrachlorobenzene¹⁹ as catalyzed by Rieske non-heme iron oxygenases involves the formation of unstable dihydrodiols (right degradative sequence), whereas attack on nonchlorinated carbon atoms give rise to stable halosubstituted dihydrodiols^{76, 121, 242} (left degradative sequence). The genetic organization of the Rieske non-heme iron oxygenases benzoate 1,2-dioxygenases of *P. putida* KT2440¹⁴⁷, 2-halobenzoate 1,2-dioxygenases of *B. cepacia* 2CBS¹¹⁰, *Burkholderia* sp. TH2³⁵⁴, *P. aeruginosa* 142³⁶⁸ and *P. aeruginosa* JB2¹³¹ and 3-chlorobenzoate 4,5-dioxygenase of *C. testosteroni* BR60²⁴³ are shown.

JB2¹³⁰, *P. aeruginosa* 142²⁹⁹ and other *Pseudomonas* sp.²⁶⁷). All these organisms catalyze a 1,2-dioxygenation of 2-chlorobenzoate thereby forming catechol (Figure 5), whereas 4-chlorocatechol is produced by successful dioxygenation of 2,4-dichloro- and 2,5-dichlorobenzoate. The only possible exception to this rule are derivatives of *B. cepacia* WR401^{75, 121}, which obviously catalyze a 1,6-dioxygenation of 2-chlorobenzoate forming 3-chlorocatechol after dehydrogenation (Figure 5). As the degradation of 4-chlorocatechol necessitates the presence of a chlorocatechol degradative pathway, it can initially be assumed, that organisms capable of degrading 2-chlorobenzoate only, and those capable of

degrading also 2,4-dichloro- or 2,5-dichlorobenzoate differ by the presence of a chlorocatechol pathway. However, two distinct 2-chlorobenzoate degrading dioxygenase systems have been described (Figure 5). The plasmid-borne two-component 2-halobenzoate 1,2-dioxygenase (oxygenase consisting of α - and β -subunits and a reductase) from strain 2CBS^{87, 110}, like the one from strain TH2³⁵⁴, is similar (56–59% of sequence identity of α -subunits) to the two-component plasmid-borne toluate 1,2-dioxygenase from *P. putida* mt-2¹¹⁹ and benzoate 1,2-dioxygenase of *P. putida* PRS2000⁵¹ and thus belongs to the family of benzoate dioxygenases¹⁰⁶. These 2-halobenzoate dioxygenases are characterized by their high activity against 2-chlorobenzoate, and also 2-fluoro-, 2-bromo-, and even 2-iodobenzoate are transformed into catechol^{87, 354}, whereas 3-chlorobenzoate is only a poor substrate. Transformation rates of 4-chloro-, 2, 4-dichloro- or 2,5-dichlorobenzoate are negligible. In contrast, the 2-chlorobenzoate dioxygenase system of *P. aeruginosa* strain 142 is a three-component dioxygenase system (oxygenase consisting of α - and β -subunits, ferredoxin and reductase)²⁹⁸. Moreover, the α -subunits of 2-chlorobenzoate dioxygenases of strains JB2 and 142³⁶⁸ exhibit only 22% of sequence identity with that of strain 2CBS, but significant levels of identity (42%) with salicylate 5-hydroxylase from *Pseudomonas* sp. strain U2⁹¹ and putative biphenyl dioxygenase α -subunits from *Novosphingobium aromaticivorans* F199³⁰⁰. Thus, 2-chlorobenzoate dioxygenases are functionally similar, but represent two different lineages with distinct activities. The dioxygenase system of strain JB2 differed only slightly from that of strain 142¹³¹. As these strains originated from California and Russia, respectively, it was suggested that *ohbAB* and/or the mobile element on which the genes are carried might have a global distribution. The most versatile chlorobenzoate degrader is *P. putida* P111¹²⁸, which also grows on 4-chlorobenzoate. Its capabilities were assumed to result from the involvement of two separate dioxygenases, a chromosomal encoded one with specificity similar to that of toluate 1,2-dioxygenase and a plasmid encoded one, with activity similar to the 2-chlorobenzoate dioxygenases of strains JB2 and 142³⁴.

Another dechlorinating system capable of dechlorinating 3-chlorosubstituted benzoates has been described in *C. testosteroni* strain BR60²⁸³. 3-Chlorobenzoate (as a nonsymmetric compound it can bind as *m*-chloro as well as *m'*-chloro analog) is dominantly subject to 4,5-dioxygenation²⁴⁰ by CbaAB (3-chlorobenzoate 4,5-dioxygenase and reductase)²⁴³ to give 5-chloroprotocatechuate after CbaC mediated dehydrogenation (Figure 5). A minor amount of 3-chlorobenzoate is 3,4-dioxygenated, resulting in an unstable dihydrodiol, which spontaneously eliminates chloride to form protocatechuate²⁴². 3,4-Dichlorobenzoate is exclusively dechlorinated to give 5-chloroprotocatechuate^{240, 242}, which is a substrate for the protocatechuate *meta*-ring fission pathway in BR60 as described by Kersten *et al.*¹⁶⁰. The oxygenase belongs to the group I dioxygenases²⁴⁴ or phthalate family of dioxygenases¹⁰⁶, a large and

diverse family of oxygenases, comprising both mono- and dioxygenases, where the oxygenase subunit has an α_n subunit configuration (in contrast to other oxygenase systems, which consist of, α - and β -subunits).

A similar dechlorinating dioxygenase, probably belonging to the phthalate family of dioxygenases²⁴³ has been described in *Pseudomonas* sp. strain CBS^{171, 213}. The enzyme system, consisting of an α_3 -oxygenase²¹⁴ and a FMN containing reductase³²⁷, converts 4-chlorophenylacetate to 3,4-dihydroxyphenylacetate (Figure 5) and exhibits high substrate specificity. Only 4-bromo- and 4-fluorophenylacetate were transformed with significant activity, whereas phenylacetate, benzoate or hydroxylated analogs were not oxidized²¹⁴.

An oxygenolytic mechanism was also reported to be responsible for dechlorination carried out by biphenyl 2,3-dioxygenase from *Burkholderia* sp. strain LB400^{113, 333} and suggested for the enzyme from *C. testosteroni* B-356³. By those enzymes, 2-chlorosubstituted biphenyls are transformed into unstable dihydroxy compounds, which spontaneously rearomatize with concomitant elimination of chloride, to give the corresponding catechol derivatives. By exchanging subunits between different dioxygenase systems, several groups have shown that the α -subunit of Rieske non-heme iron oxygenases is dominantly responsible for substrate specificity^{20, 81, 98, 136, 265, 359}. Despite major differences in substrate specificity and dehalogenation the α -subunits of, for example, *Pseudomonas pseudoalcaligenes* KF707 and *Burkholderia* sp. LB400 are nearly identical. Determinants of substrate selectivity and regioselectivity of dioxygenation have been discovered^{81, 165, 231}, and only few amino acid changes resulted in proteins with significantly altered properties. Thus, even though only the LB400 derived biphenyl dioxygenase was thus far shown to be capable of catalyzing dehalogenation, it can be assumed that such a capacity is not restricted to certain species, but possibly spread among organisms carrying biphenyl dioxygenase genes, including *Pseudomonas* sp.

A further dioxygenase capable of carrying out oxygenolytic dehalogenation is the tetrachlorobenzene dioxygenase of *Ralstonia* sp. strain PS12^{19, 309}. This dioxygenase differs from the above-described dehalogenating dioxygenases, as lower chlorinated derivatives harboring two unsubstituted carbon atoms are subject to a conventional dioxygenation resulting in dihydrodiols¹⁹, and only 1,2,4,5-tetrachlorobenzene, which misses two neighbored unsubstituted carbon atoms, is subject to oxygenolytic dehalogenation (Figure 5). Sequential exchange of individual amino acids in the highly homologous nondehalogenating toluene dioxygenase of *P. putida* F1 identified a single amino acid residue in the α -subunit of the dioxygenase as critical for dehalogenation²⁰. Since the bulkier methionine is located at this position in nondehalogenating Tod dioxygenase and the less bulky alanine is present in the dehalogenating Tec dioxygenase, it seems likely that the larger halogenated substrate is sterically hindered by the methionine from entering the catalytic

site. Enzyme variants could be obtained, which also dehalogenated 2,4,5-trichlorotoluene²⁸⁰. Further experiments (Beil, unpublished) indicated that the chlorobenzene dioxygenase of *Pseudomonas* sp. strain P51 also is capable of dehalogenating 1,2,4,5-tetrachlorobenzene.

4. METABOLISM OF CHLOROAROMATICS BY CENTRAL CATABOLIC ROUTES

4.1. The 3-Oxoadipate Pathway

The aerobic degradation of aromatic compounds usually involves their successive activation and modification such that they are channeled toward a few dihydroxylated intermediates such as catechol, gentisate or protocatechuate (Figure 3), which are then subject to ring cleavage. Many of the enzyme systems capable of activating aromatic compounds are of broad substrate specificity and transform chlorinated substrate analogs, often resulting in the formation of chlorinated catechols (see Section 3.1). However, only a small fraction of bacteria able to transform chloroaromatics is capable of mineralizing them, as this usually requires the presence of enzymes of the chlorocatechol pathway (see Section 2). Chlorocatechols can be regarded as environmentally important intermediates and their metabolic fate when processed by enzymes of widespread pathways for the metabolism of catechol can be of environmental significance. The chromosomal encoded 3-oxoadipate pathway is such a pathway that is widely distributed in soil bacteria and fungi¹²². One branch of this pathway converts protocatechuate (Figure 6), derived from phenolic compounds including *p*-cresol, 4-hydroxybenzoate and numerous monomers formed from lignin breakdown, whereas the other branch converts catechol (Figure 6), generated from various aromatic hydrocarbons, amino aromatics, and lignin monomers. Two additional steps, a 3-oxoadipate:succinyl-CoA transferase and a 3-oxoadipyl-CoA thiolase accomplish the conversion of 3-oxoadipate to tricarboxylic acid cycle intermediates³⁴⁹. Enzyme studies and amino acid sequence data indicate that the pathway is highly conserved in diverse bacteria, including *Pseudomonas*. The catechol branch comprises a catechol 1,2-dioxygenase (CatA), a muconate cycloisomerase (CatB), and a muconolactone isomerase (CatC), whereas the protocatechuate branch comprises a protocatechuate 3,4-dioxygenase (PcaGH), a carboxymuconate cycloisomerase (PcaB) and a carboxymuconolactone decarboxylase (PcaC). Both branches thus converge at the stage of 3-oxoadipate enol-lactone, which is transformed by an enol-lactone hydrolase (CatD, PcaD) into 3-oxoadipate, which is transformed by CatIJ/PcaIJ and CatF/PcaF into TCA cycle intermediates.

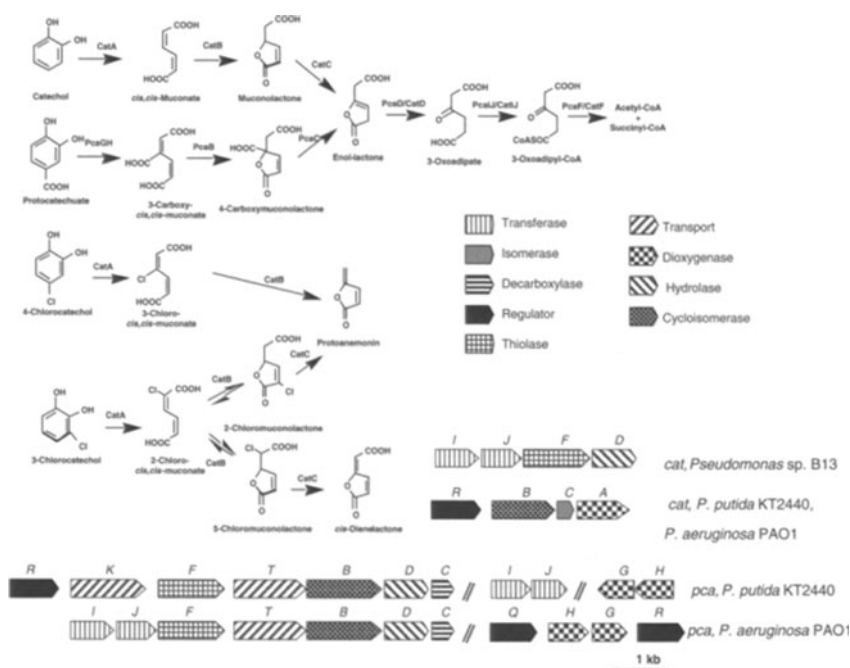


Figure 6. Catechol and protocatechuate branches of the 3-oxoadipate pathway and metabolic fate of 3-chloro-^{284, 341, 381} and 4-chlorocatechol²⁷ by enzymes of the catechol pathway. The organization of gene clusters for protocatechuate (*pca*) and catechol (*cat*) metabolism in *P. putida* KT2440^{147, 245}, *P. aeruginosa* PAO1^{147, 351} and *Pseudomonas* sp. B13¹⁰⁷ are shown. *catA*, catechol 1,2-dioxygenase gene; *catB*, muconate cycloisomerase gene; *pcaB*, carboxymuconate cycloisomerase gene; *catC*, muconolactone isomerase gene; *pcaC*, carboxymuconolactone decarboxylase gene; *catD* and *pcaD*, 3-oxoadipate enol-lactone hydrolase genes; *catF* and *pcaF*, thiolase genes; *pcaHG*, protocatechuate 3,4-dioxygenase genes; *pcaIJ* and *catIJ*, transferase genes; *pcaK* and *pcaT*, transport genes; *pcaR* and *pcaQ*, regulator genes.

cat genes and *pca* genes are found in various *Pseudomonas* strains and overviews on the genetic organization have been published^{107, 122, 147}.

It has recently been shown that there are severe differences between reactions catalyzed by the chlorocatechol and the 3-oxoadipate pathway enzymes. In both cases, chlorocatechols were subject to intradiol cleavage with the corresponding *cis,cis*-muconates as products^{27, 68, 69}. However, muconate and chloromuconate cycloisomerases perform distinct reactions. Whereas chloromuconate cycloisomerases catalyze a dehalogenation of 3-chloro-*cis,cis*-muconate to form *cis*-dienelactone (Figure 1), muconate cycloisomerases catalyze a dehalogenation and decarboxylation to form protoanemonin (Figure 6)²⁷, a compound of high toxicity³³¹. Protoanemonin formation was observed to be obviously a common reaction performed

by proteobacterial muconate cycloisomerases, including those from *Pseudomonas* strains³⁷⁷. Protoanemonin formation in turn was assumed to be the reason for the poor survival of PCB cometabolizing organisms in soil microcosms due to channeling of intermediary chlorobenzoate into the 3-oxoadipate pathway²⁶. Even chloroprotoanemonin was reported to be formed from 2,4-dichloromuconate by *P. putida* muconate cycloisomerase¹⁵⁹. Also in the case of 2-chloromuconate turnover, muconate and chloromuconate cycloisomerases were shown to catalyze different reactions. Whereas chloromuconate cycloisomerase catalyzes dehalogenation to form *trans*-dienelactone (Figure 1), obviously via 5-chloromuconolactone as an intermediate³⁷⁹, muconate cycloisomerases catalyze cycloisomerization only, to form both 2-chloro- and 5-chloromuconolactone (Figure 6) as stable products³⁸¹. Only chloromuconate cycloisomerase, but not muconate cycloisomerase, catalyzes dehalogenation of 5-chloromuconolactone^{379, 381}. However, it has recently been shown that 5-chloromuconolactone is a substrate of proteobacterial muconolactone isomerases^{284, 285} of the 3-oxoadipate pathway. Muconolactone isomerase catalyzes a dehalogenation of 5-chloromuconolactone to form *cis*- and *trans*-dienelactone in a 3-4:1 ratio. The mechanism was proposed via abstraction of the C4 proton followed by spontaneous chloride elimination. The metabolism of 2-chloro-*cis,cis*-muconate via the 3-oxoadipate pathway should then, taking into account the equilibrium between 2-chloro- and 5-chloromuconolactone, and 2-chloro-*cis,cis*-muconate catalyzed by muconate cycloisomerase³⁸¹, result in the formation of dominantly *cis*-dienelactone from 2-chloromuconate (Figure 6). However, like 5-chloromuconolactone, also 2-chloromuconolactone harbors a proton at C4, which can be abstracted by muconolactone isomerase. Protoanemonin was shown to be formed (Figure 6), probably by elimination of CO₂ and chloride from chlorosubstituted 3-oxoadipate enol-lactone³⁴¹.

Specific activities of 3-oxoadipate pathway enzymes were also realized for 4-fluorocatechol turnover and 4-fluoromuconolactone was observed as cycloisomerization product by muconate cycloisomerases³¹⁶. Initial evidence was given that 3-oxoadipate enol-lacton hydrolases can defluorinate this compound with formation of maleylacetate³¹⁶.

4.2. Alternative Central Pathways for Chlorinated 1,2-Diphenolic Intermediates

For a long time the presence of a catechol *meta*-cleavage pathway was assumed to severely interfere with the degradation of chloroaromatics. Catechol *meta*-cleavage routes are widespread in *Pseudomonas* and usually involved in the degradation of methylsubstituted compounds such as toluene or methylphenols^{15, 339, 394, 414}. One of the reasons of interference was assumed

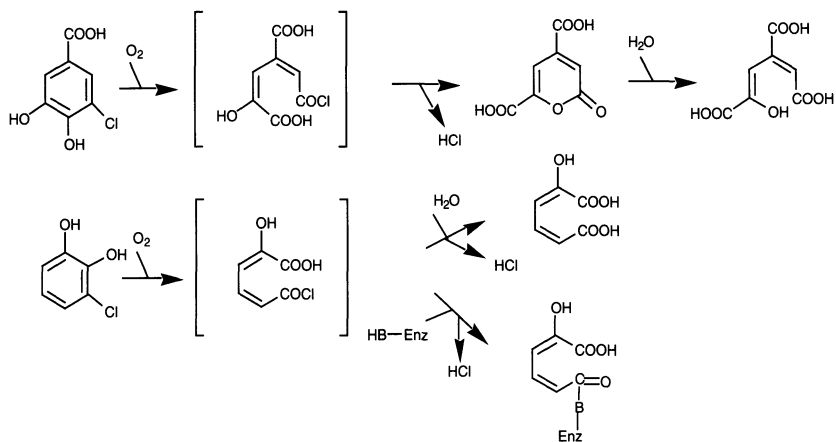


Figure 7. Meta-cleavage pathways for 5-chloroprotocatechuate (top) and 3-chlorocatechol (bottom). Acylchlorides are shown in brackets. Intramolecular rearrangement results in the formation of 2-pyrone-4,6-dicarboxylate from 5-chloroprotocatechuate¹⁶⁰, which is subject to hydrolysis by a hydrolase²¹⁷. The 3-chlorocatechol ring-cleavage product can react either with the ring cleavage enzyme resulting in suicide inactivation¹⁴, or be rapidly hydrolyzed to give 2-hydroxymuconate¹⁵⁵.

to be the formation of a suicide product, reactive acyl chloride (Figure 7), for example, from 3-chlorocatechol by the catechol 2,3-dioxygenase of *P. putida* mt-2¹⁴. Their formation results in irreversible inactivation of the ring-cleavage enzyme. Other enzymes such as the catechol 2,3-dioxygenase of *P. putida* F1 were reported to be reversibly inactivated by 3-chlorocatechol which has been attributed to the potential of the substrate to chelate the active site ferrous ion¹⁷². However, in other cases reversible inactivation was shown to be due to a rapid oxidation of the active site ferrous iron into its ferric form with concomitant loss of activity³⁷⁰ and a general mechanism for the inactivation of extradiol dioxygenases during catalytic turnover involving the dissociation of superoxide from the enzyme-catecholic-dioxygen ternary complex was suggested.

In contrast to 3-chlorocatechol, 4-chlorocatechol is a moderate substrate for various catechol 2,3-dioxygenases^{14, 167, 239}, among them catechol 2,3-dioxygenases of family I.2.A⁷⁴, which have been widespread observed to be involved in the degradation of methylaromatics by *Pseudomonas* sp. However, despite high sequence identity, members of this subfamily exhibit very different substrate specificities, and a protein 94% homologous to XylE exhibit only negligible activity with 4-chlorocatechol¹⁶⁷. Thus, the capability to transform 4-chlorocatechol is not a general characteristic of catechol 2,3-dioxygenases of family I.2.A. 4-Chlorocatechol is also a poor substrate for the 2,3-dioxygenase TodF of *P. putida* F1, which belongs to subfamily I.3.B and is thus, related to dioxygenases with high activity against bicyclic substrates (2,3-dihydroxybiphenyl). Actually, enzymes related to TodF were shown to be highly active

with 2,3-dihydroxybiphenyl²¹. Like for other biphenyl 2,3-dioxygenases^{167, 222}, substitution at the 3-position seems to be essential for activity. Some more recent publications postulated that compounds degraded via catechols chlorinated in the 4-position might be mineralized via a catechol degrading *meta*-cleavage pathway^{6, 7, 134, 138, 139, 145, 219} but information is missing about the way in which the products are dechlorinated.

Recently, *P. putida* GJ31 was found to degrade chlorobenzene rapidly via 3-chlorocatechol and uses a *meta*-cleavage pathway²¹⁵. In contrast to other catechol 2,3-dioxygenases, which are subject to inactivation, the chlorocatechol 2,3-dioxygenase of strain GJ31 productively converts 3-chlorocatechol^{155, 216}. Stoichiometric displacement of chloride occurs leading to the production of 2-hydroxymuconate (Figure 7), which is further converted through the *meta*-cleavage pathway.

A productive *meta*-cleavage without suicide effect is known for about 20 years for the degradation of 5-chloroprotocatechuate (Figure 7). Protocatechuate is a central intermediate in the degradation of various carboxylated aromatic compounds such as vanillate, vanillin, caffeate or *p*-coumarate. Three modes of further degradation of protocatechuate have been reported. The intradiol cleavage by a protocatechuate 3,4-dioxygenase seems to be widespread in *Pseudomonas* strains, for example, a collection of more than 100 *Pseudomonas* strains displayed such an activity³⁴⁸ and respective *pca* gene clusters were localized in the genomes of *P. putida* KT2440, *P. aeruginosa* PAO1, *P. fluorescens* Pfo-1 and *P. syringae* pv. tomato DC3000¹⁴⁷. However, this pathway seems not to be suited for the degradation of chloroprotocatechuate. Alternative pathways are the *meta*-cleavage pathways. Whereas a protocatechuate 2,3-dioxygenase has so far only been described from a *Bacillus* isolate³⁹³, protocatechuate 4,5-dioxygenase seems to be widespread, and *Delftia acidovorans* or *C. testosteroni* and also *Sphingomonas paucimobilis* strains usually exhibit such an activity^{250, 348}, whereas *Pseudomonas* isolates or *B. cepacia* do not³⁴⁸. However, genes similar to those encoding the protocatechuate 4,5-dioxygenase pathway were observed in the genome of strain KT2440, but the functioning of this pathway has not been proven yet¹⁴⁷. Only the protocatechuate 4,5-dioxygenase pathway has been shown to be functional for the degradation of chloroprotocatechuate and the formation of 2-pyrone-4,6-dicarboxylate by nucleophilic displacement of a halide ion from protocatechuates substituted with a halogen at the C5 of the nucleus was shown^{160, 161} (Figure 7). This indicated that cyclization entailing nucleophilic displacement of halogen provides an effective alternative to the enzyme suicide inactivation that occurs when a nucleophilic group of the dioxygenase undergoes acylation. An important aspect of this mechanism is that the ring fission product remains bound to the enzyme during a complete configuration change that precedes nucleophilic displacement. Hydrolysis of the pyrone is followed by degradation through this *meta*-cleavage pathway.

5. THE HYDROQUINONE PATHWAYS

There are numerous reports of diverse bacteria degrading pentachlorophenol (PCP), and literature suggested identification as *Arthrobacter* sp. strain ATCC33790⁷¹, *Flavobacterium* sp. strain ATCC 39723⁵², *Pseudomonas* sp. strain SR3²⁹⁶, *Pseudomonas* sp. RA2²⁸⁶ and *Pseudomonas saccharophila* strains KF1T, KF3 and NKF1²⁵¹. However, detailed taxonomic work had shown later that all those organisms were closely related and represent a monophylogenetic group. 16S rRNA analysis together with fatty acid and sphingolipid analyses strongly suggested that these strains are members of what at that time was called the genus *Sphingomonas*^{153, 251}. Thus, it was suggested that PCP degradation by Gram-negative bacteria might occur only in the genus *Sphingomonas* and that *Pseudomonas* strains are not involved in PCP degradation.

The biochemistry (Figure 8) and genetics of PCP degradation have been most intensively studied in *Sphingobium chlorophenolicum* ATCC39723 (previously *Sphingomonas chlorophenolica* or *Flavobacterium* sp.^{305, 356}). PCP degradation is initiated by pentachlorophenol 4-monooxygenase (PcpB)^{260, 397}. In contrast to previous assumptions of tetrachloro-*p*-hydroquinone to be the reaction product³⁹⁷, Dai *et al.*⁵⁶ showed that tetrachlorobenzoquinone is the reaction product, which is reduced in a NADPH-dependent reduction into tetrachlorohydroquinone by the *pcpD* gene product, tetrachlorobenzoquinone reductase. Tetrachlorohydroquinone reductive dehalogenase PcpC catalyzes the glutathione dependent reductive dehalogenation first to trichlorohydroquinone and then to 2,6-dichlorohydroquinone^{259, 398}. 2,6-Dichlorohydroquinone is degraded by PcpA, which, in contrast to previous assumptions¹⁹¹, is a dioxygenase, and not a hydrolytic dehalogenase³⁹⁶. Evidence for the formation of 2-chloromaleylacetate was given^{255, 400}, which should arise after hydrolysis of the direct ring-cleavage product. Maleylacetate reductase, as shown for the degradation of chlorocatechols, is assumed to channel 2-chloromaleylacetate into the Krebs cycle, and a maleylacetate reductase encoding gene *pcpE* was localized upstream of *pcpA*⁴⁰. Interestingly, the genes *pcpA*, *pcpBD*, *pcbC* and *pcpE* were found at four discrete locations and whereas *pcbC* was constitutively expressed, the other genes were inducible⁴⁰.

PcpB carries out the initial attack on PCP. Its gene, *pcpB*, has been sequenced from various *Sphingomonas* isolates and *pcpB* sequences were identical in strains ATCC 39723, SR3 and RA2⁷⁰. Highly homologous genes were observed in other sphingomonads^{70, 196, 363}. Distribution of the *pcpB* gene was studied in a phylogenetically diverse group of PCP degraders isolated from contaminated groundwater in Finland²¹². Surprisingly, highly identical *pcpB* alleles, but distinct from previously identified ones, were determined in all PCP degrading sphingomonads, suggesting a recent transfer of this gene

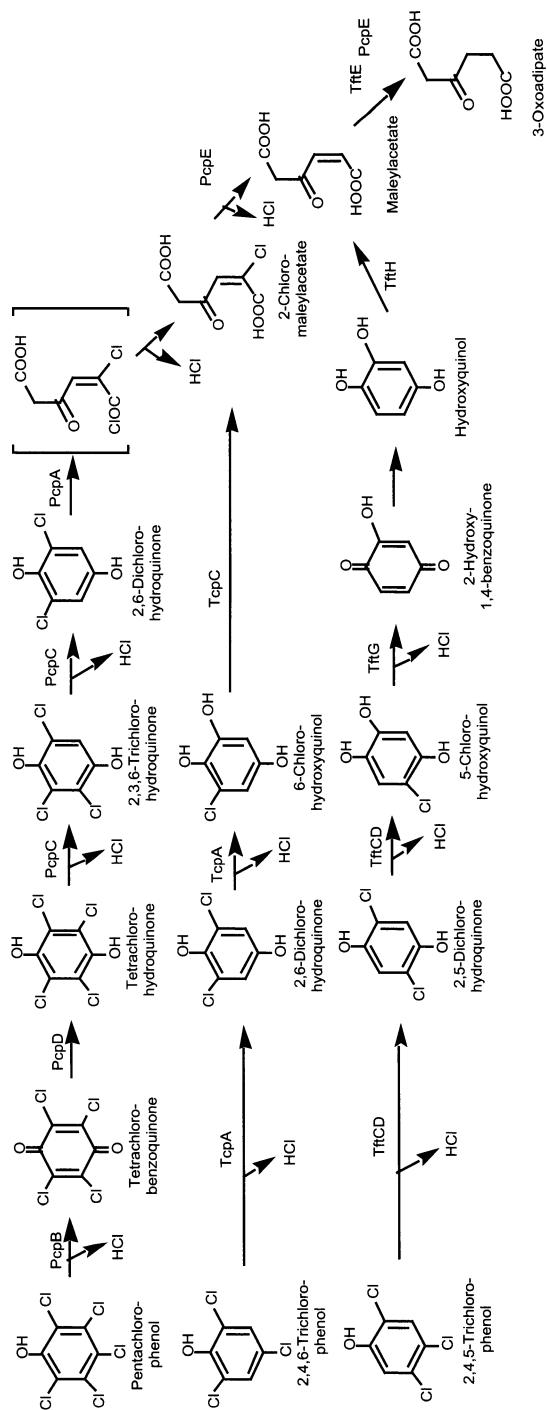


Figure 8. Degradation of pentachloro-^{56, 396-398}, 2,4,6-trichloro-^{208, 262} and 2,4,5-trichlorophenols^{58, 399, 410} via hydroquinones as central intermediates.

in situ in the groundwater³⁶⁴. However, *pcpB* genes were not observed in other proteobacteria, including a *Pseudomonas* strain, even if they were capable of PCP transformation. Thus, such organisms, including the *Pseudomonas*, should harbor a different genetic equipment responsible for polychlorophenol degradation. However, *pcpB* gene sequences have been observed in organisms not capable of PCP depletion, among them a *Pseudomonas* isolate³⁰⁶. It was suggested that the presence of a *pcpB* gene only in *Pseudomonas* does not necessarily confer to them the capability to transform PCP. Still, there are reports on PCP degrading *Pseudomonas* and a detailed phylogenetic study was performed on the PCP degrading strain *Pseudomonas* sp. Bu34. Even though catabolic genes or enzymes were not described¹⁹³, that study shows that PCP degrading *Pseudomonas* strains exist in nature, however, their degradation pathways and their environmental importance remain to be elucidated.

Chlorophenol monooxygenases not related to *pcpB* have been reported to be involved in 2,4,5-trichlorophenoxyacetate (2,4,5-T) or 2,4,6-trichlorophenol degradation (Figure 8), and genes have thus far been described from members of the genera *Ralstonia*^{208, 262, 357} and *Burkholderia*³⁹⁹. At least the enzymes isolated from *R. pickettii* DTP0602 and from *B. cepacia* AC1100 exhibited some PCP transforming activity. Whereas the *R. pickettii* enzyme (like the one isolated from *Azotobacter* sp. strain G1³⁸⁶) was shown to produce 2,6-dichlorohydroquinone, the monooxygenases of strains *R. eutropha* JMP134 and *B. cepacia* AC1100 obviously act twice on the substrate, forming monochlorohydroquinones from trichlorophenols. However, such genes have thus far not been reported to be present in *Pseudomonas*.

Above-mentioned pathways for polychlorophenol degradation differ evidently in the intermediate being subject to ring cleavage. Whereas in PCP degrading sphingomonads, 2,6-dichlorohydroquinone is the ring-cleavage substrate⁴⁰⁰, 6-chlorohydroxyquinol is the ring-cleavage substrate in 2,4,6-trichlorophenol degradation by strain JMP134²⁰⁸, while hydroxyquinol is the ring-cleavage substrate in 2,4,5-trichlorophenoxyacetate degradation by *B. cepacia* AC1100⁴¹⁰. Thus, in the *Sphingomonas* pathway three chloride molecules are eliminated before ring-cleavage, two of them by tetrachlorohydroquinone dehalogenase³⁹⁸.

The enzymes responsible for 2,6-dichlorohydroquinone ring-cleavage are special in that they attack an aromatic ring with two hydroxyl groups *para* to each other²⁵⁵. The *pcpA* gene products show high similarity to *linE* involved in the degradation of hexachlorocyclohexane by *S. paucimobilis*²²⁶ and another putative hydroquinol *meta*-cleavage dioxygenase from plasmid pNB1 of an organism tentatively identified as *P. putida*²⁶⁶. However, respective enzymes are obviously not restricted to PCP degrading sphingomonads. A strain designated *Pseudomonas* sp. HH35 and capable of degrading 3,5-dichloro- and 3,5-dibromo-4-hydroxybenzoate among other compounds was

shown to be capable of directly cleaving methyl- or chlorosubstituted hydroquinones³²⁰ and thus to harbor an enzyme similar in activity to that observed in PCP degrading sphingomonads.

In contrast, degradation of 2,4,5-trichloro- and 2,4,6-trichlorophenol occurs via ring-cleavage of trihydroxylated intermediates (Figure 8). Whereas 6-chlorohydroxyquinol formed from 2,4,6-trichlorophenol can be directly subject to ring-cleavage forming 2-chloromaleylacetate²⁰⁸ in *B. cepacia* AC1100, 5-chlorohydroquinone is converted to hydroxybenzoquinone by TftG dechlorinase and hydroxyquinol formed after hydrobenzoquinone reductase mediated reduction is subject to ring cleavage by TftH⁴¹⁰. (Chloro)hydroxyquinol dioxygenases form a defined phylogenetic group comprising also the enzymes of *Sphingomonas wittichii* RW1¹⁰, *Arthrobacter* sp. strain BA-5-17²³⁷ and *R. pickettii* DTP0602³⁵⁷. Despite the availability of only few biochemical data it is evident that members of this family differ in substrate specificity toward chlorinated substrate analogues^{58, 189, 208}. How far such ring-cleavage enzymes are spread in *Pseudomonas* is not known.

6. 4-CHLOROBENZOATE HYDROLYTIC DEHALOGENATION

The degradation of 4-chlorobenzoate by a pathway distinct from that via chlorocatechol, but involving an early dehalogenation (Figure 9), was indicated as early as 1976³⁰⁴. Strains of different genera degrading 4-chlorobenzoate by an obviously similar mechanism have been isolated^{169, 170, 304}. *Pseudomonas* sp. CBS3 can be regarded as the archetype organism from

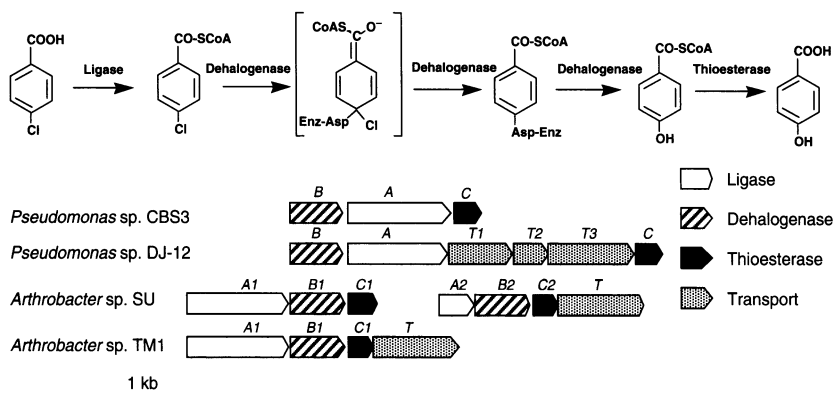


Figure 9. Hydrolytic dehalogenation of 4-chlorobenzoate^{44, 203, 206} and organization of the gene clusters in *Pseudomonas*^{12, 43} and *Arthrobacter*^{102, 322} strains.

which the metabolic route and the mechanism of hydrolytic dehalogenation have been elucidated in detail²³⁵. The 4-chlorobenzoate dehalogenase system from *Pseudomonas* sp. strain CBS3 has been shown to be a three-component enzyme complex^{73, 205}, and the role of each component has been clarified^{312, 323} (Figure 9). The activation of the substrate is carried out by an ATP-dependent 4-halobenzoate-coenzyme A ligase^{44, 204, 206} which shares significant sequence similarity with proteins which catalyze similar chemistry in the β -oxidation pathway¹². The activation reaction precedes dehalogenation, which is catalyzed by a 4-halobenzoyl-CoA dehalogenase forming 4-hydroxybenzoyl-CoA^{199, 203} in a hydrolytic substitution reaction. Substrate binding to the dehalogenase active site is followed by nucleophilic attack of the carboxylate side chain of Asp145 at the C4 of the benzoyl group^{53, 200, 404} to form a Meisenheimer intermediate⁶⁶. The chloride is displaced from this intermediate because of rearomatization of the benzoyl-group to form an enzyme-ester and hydrolysis of the ester generates the 4-hydroxybenzoyl-CoA product. This enzyme was reported to share ancestry with the 2-enoyl-CoA hydratase family^{12, 395}. The last step in the reaction to form 4-hydroxybenzoate is carried out by the 4-hydroxybenzoate:coenzyme A thioesterase. The absence of serine, cysteine or histidine catalytic residues distinguish this protein from all other thioesterases characterized to date^{12, 22}. However, crystallographic investigation revealed a high similarity of its three-dimensional structure to that of β -hydroxydecanoyl thiol ester dehydrase from *E. coli*¹⁹⁴, and by superimposing the two structures, it has been possible to identify the possible active site region and to propose a catalytic mechanism whereby the carboxylate side chain of Asp17 activates a water molecule for subsequent nucleophilic attack at the thioester carbonyl carbon of the substrate²².

The genes encoding the 4-chlorobenzoate dehalogenase system (Figure 9) were reported to be chromosomal localized and organized in an operon in the *fcfB* (dehalogenase), *fcfA* (ligase), and *fcfC* (thioesterase) order¹². The gene organization in a second 4-chlorobenzoate-degrading *Pseudomonas* sp. strain DJ-12 was similar, however, with the addition of three new genes, localized inbetween *fcfA* and *fcfC* and supposedly responsible for 4-chlorobenzoate transport⁴³. Additional information on the genetic organization of 4-chlorobenzoate dehalogenase genes is available from an *Alcaligenes* sp. AL3007 and three *Arthrobacter* species (TM1, SU and FHP1)^{102, 322}. Whereas the *Alcaligenes* operon shows a structure similar to that of above *Pseudomonas* strains, the structure in 4-chlorobenzoate degrading *Arthrobacter* strains is different (Figure 9). All those strains contain at least one *fcf* gene cluster organized in an *fcfABC* order, followed by an ORF probably encoding a 4-chlorobenzoate transporter. The deduced protein sequences of the *Arthrobacter* 4-chlorobenzoate dehalogenase genes share high sequence similarity (>97%, 95% and 78% amino acid identity for FcbA, B and C, respectively), and are only

distantly related to the proteobacterial 4-chlorobenzoate dioxygenase (amino acid sequence identity of >47% and 43% for FcbA and FcbB, respectively, no significant similarity was observed between FcbC from *Arthrobacter* strains and from Proteobacteria). Despite the similar gene arrangement in proteobacteria, FcbA and FcbC of strain CBS3 exhibit only low identity to the highly similar FcbA and FcbC of AL3007 (AF537222) and DJ-12⁴³. No rule can be drawn for the localization of 4-chlorobenzoate dehalogenase genes. They are obviously chromosomal encoded in strains CBS3, DJ12 and TM1, but plasmid encoded in strains AL3007 and SU⁴³.

Already early reports indicated, that chlorobenzoate hydrolytic dehalogenating activity is restricted to 4-halosubstituted benzoates, and strain CBS3 was shown to grow on 4-chloro- and 4-bromo- but not 4-fluorobenzoate¹⁷⁰, indicating the nature of the halogen substituent also to be of importance for enzyme activity. Similarly, both 4-chloro- and 4-bromobenzoate and even 4-iodobenzoate were dehalogenated by cell extracts of *Arthrobacter* sp. SU²³⁴, indicating similar substrate specificity of the different dehalogenase enzyme systems. The substrate specificity determinants were later analyzed using the strain CBS3 dehalogenase system, which was also shown to be capable of dehalogenating 4-iodobenzoate³⁶¹. The ligase was capable of transforming all 4-halobenzoates with high rates²⁰⁶, whereas 2-halo- or 3-halobenzoates were not transformed. Differences in transformation were, however, observed for 4-halobenzoyl-CoAs by FcbB. Whereas the chloro-, bromo-, and iodo-derivatives were transformed at high rates, the turnover rate for 4-fluorobenzoyl-CoA was five orders of magnitude lower¹⁹⁹. Obviously, the formation of the arylated enzyme by halide elimination from the Meisenheimer complex is hindered in case of the 4-fluoro-derivative. The failure to effectively transform 4-fluorobenzoate seems to be a common feature of the dehalogenase enzyme system of most organisms analyzed in this respect so far^{177, 372}. However, Oltmanns *et al.*²⁵⁷ reported on an *Aureobacterium* isolate capable of degrading 4-fluorobenzoate via 4-hydroxybenzoate, but not capable of growing on 4-chlorobenzoate.

7. METABOLISM OF CHLORINATED BICYCLIC AROMATICS

7.1. Metabolism of Chlorobiphenyls

Among chlorinated aromatics, the degradation of bicyclic compounds such as PCBs or chlorinated dioxins received special attention. A number of biphenyl-degrading organisms have been isolated and they are commonly

capable of transforming PCB congeners. These organisms belong to both Gram-negative and Gram-positive genera and comprise various *Pseudomonas* isolates (such as *P. pseudoalcaligenes* KF707³⁵⁵, *P. putida* KF715¹²⁵, *P. putida* OU83¹⁶³, *Pseudomonas* sp. strain KKS102⁹⁴) and catabolize biphenyl to benzoate and 2-hydroxypenta-2,4-dienoate via the so-called upper pathway consisting of four enzymes (Figure 10): biphenyl 2,3-dioxygenase (BphA), 2,3-dihydro-2,3-dihydroxybiphenyl 2,3-dehydrogenase (BphB), 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC), and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (BphD)³².

To a significant extent, the spectrum of PCB congeners that can be transformed by an organism is determined by the specificity of the biphenyl 2,3-dioxygenase, the enzyme which catalyzes the first step in the upper pathway and which, as the toluene dioxygenase of *P. putida* F1 belongs to the toluene/biphenyl family of Rieske type non-heme iron dioxygenases. Studies on various biphenyl 2,3-dioxygenases have revealed considerable differences in their congener selectivity patterns, as well as their preference of the attacked ring^{81, 165, 223, 334, 413}. The different substrate specificities in these strains were due to relatively few differences in *bphA*, the gene coding the large subunit of the terminal biphenyl dioxygenase. Site-directed mutagenesis and gene shuffling methods were applied to construct enzymes combining the broad congener specificity of strain LB400 with increased activity against congeners oxidized especially by the strain KF707-dioxygenase^{81, 97, 353}.

Though certain PCBs serve as substrates for biphenyl dioxygenases, PCB-degrading organisms do not usually use PCBs as an energy source, but rather cometabolically catabolize the substrates. Not surprisingly, metabolites of the upper pathway may be formed as dead-end products^{17, 100, 332}. Knowledge of metabolic activities of upper pathway enzymes downstream of biphenyl dioxygenase is of interest to further understanding of the capacity of upper pathways. As an example Seah *et al.*^{329, 330} have shown that the *bphD* gene products of *Burkholderia* sp. strain LB400 and that of *Rhodococcus globerulus* strain P6 differ significantly in their kinetic properties for chlorinated substrate derivatives, however, no detailed analysis has been performed thus far on other *Pseudomonas* derived enzymes. Significant differences were also observed in substrate specificities of *bphC* gene products. Usually, extradiol dioxygenases involved in biphenyl degradation belong to the I.3.A subfamily of extradiol dioxygenases⁷⁴ and are specialized for transformation of 2,3-dihydroxybiphenyls. Those enzymes differ in substrate specificity, but generally seem to be capable of transforming various chlorosubstituted derivatives^{57, 126}. A new class of single domain extradiol dioxygenases, thus far observed only in *Sphingomonas*¹²⁷ and *Rhodococcus* strains^{11, 308}, however, has been shown to be specially adapted to the transformation of 2-chlorosubstituted 2,3-dihydroxybiphenyls^{222, 369}. Thus, interpretation of the potential for

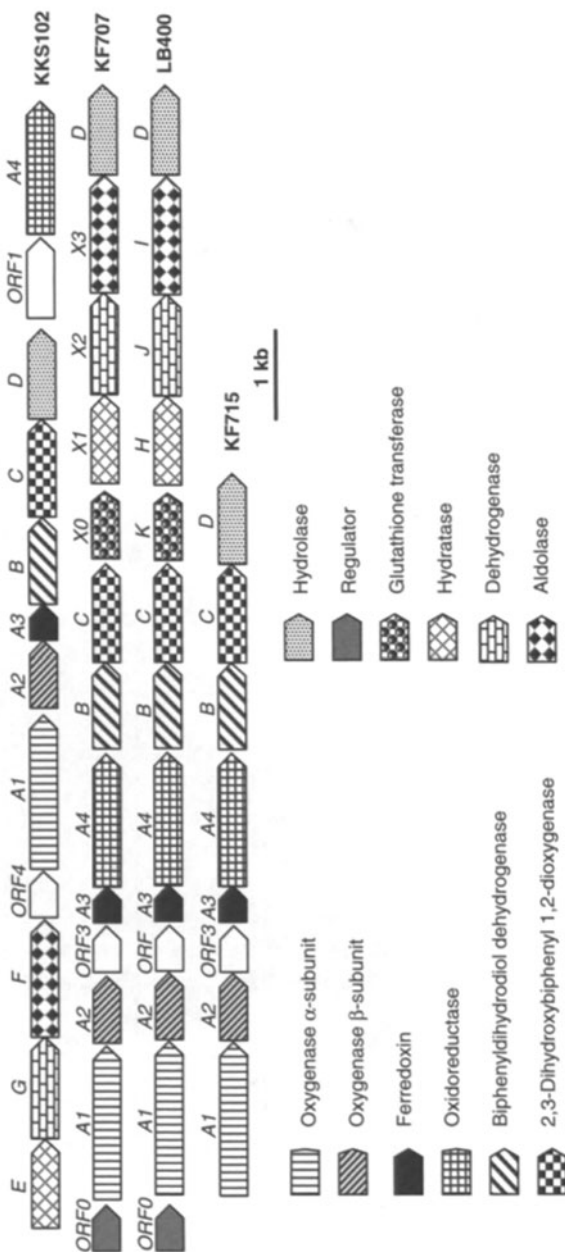
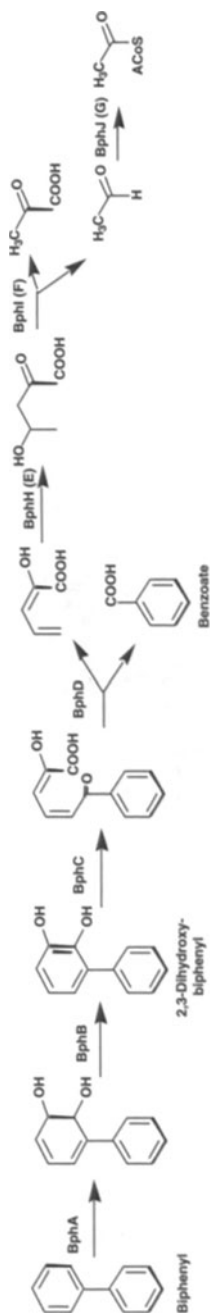


Figure 10. Pathway for biphenyl degradation and genetic organization of the *bph* gene clusters of *Pseudomonas* sp. KKS102⁹⁴, *P. pseudoalcaligenes* KF707³⁵⁵, *Burkholderia* sp. LB400³⁷ and *P. putida* KF715¹²⁵.

transformation of PCBs may be complicated by the existence of isoenzymes. *Bph* genes are usually organized in gene clusters and have been localized on bacterial chromosomes^{82, 99}, plasmids¹⁴¹ and transposons³⁴⁶. Two major variants of this gene clusters are known (Figure 10). Organisms like *P. putida* KF715 contain a *bphABCD* gene cluster. In strain LB400, genes encoding a glutathione S-transferase and enzymes involved in the transformation of the C-5 compound released during hydrolysis of the ring-cleavage product to form benzoate are localized between *bphC* and *bphD*¹³⁷. A more detailed analysis on various biphenyl degrading isolates revealed that such an organization like in strain LB400 is not unique¹³ but occurs also in *R. eutropha* H850¹⁸, *P. pseudoalcaligenes* KF707 and various *Pseudomonas agarici*, *Pseudomonas oleovorans*, *Pseudomonas balearica* and *P. putida* isolates and thus seems to be widespread in *Pseudomonas*. The presence of similar genes in different strains implies that even the chromosomal *bph* genes possess or have used mechanisms for mobilization in other strains²⁴⁹.

Even though organisms degrading biphenyl usually only cometabolize chlorinated derivatives, microorganisms capable of mineralizing them have been obtained by judicious combination of pathway segments comprising the biphenyl upper pathway, a benzoate/toluuate or 2-chlorobenzoate dioxygenase system and a chlorocatechol pathway^{124, 129, 219, 230}, usually by in vivo conjugative mating of appropriate strains. Among these, the hybrids *B. cepacia* JHR22 and *Pseudomonas* sp. UCR2 have been reported to grow on environmentally important 2-, 2,4- and 2,5-substituted PCB congeners^{124, 129}. Use of the *Pseudomonas* derived chlorobenzoate dehalogenase genes like 4-chlorobenzoate dehalogenase resulted in highly efficient mineralization pathways, primarily due to circumvention of forming toxic chlorocatechols¹⁴².

7.2. Transformation of Biarylethers

Studies by Cerniglia *et al.*⁴² and Klecka and Gibson^{173, 174} using naphthalene or biphenyl degrading *Pseudomonas* or *Sphingomonas* strains indicated that dibenzo-*p*-dioxin, dibenzofuran and chlorinated derivatives were transformed into dead-end products. The substrates were attacked, in analogy to the biphenyl or naphthalene transformation, at the lateral 1,2- and 2,3-positions, giving rise to dihydrodiols, which were subsequently dehydrogenated to dihydroxy-compounds, and, in case, subject to ring-cleavage (Figure 11). Whereas such a lateral dioxygenation is appropriate to initiate degradation of biphenyl, and naphthalene, various authors have shown that it is inappropriate for the degradation of biarylethers^{25, 297, 336}. The analysis of the bacterial degradation of dibenzofuran^{80, 88}, carbazole³¹¹, dibenzo-*p*-dioxin³⁹¹ and diphenyl ether^{61, 321}, revealed the presence of a novel mode of dioxygenation reaction for the aromatic nucleus, termed angular dioxygenation (Figure 11). Here, the

carbon bonded to the heteroatoms, and the adjacent carbon in the aromatic ring are oxidized. Angular dioxygenation of dibenzofuran, carbazole, dibenzo-*p*-dioxin and diphenyl ether produces chemically unstable hemiacetal-like intermediates, which are spontaneously converted to 2,2',3-trihydroxybiphenyl, 2'-amino-2,3-dihydroxybiphenyl, 2,2',3-trihydroxybiphenyl ether and phenol plus catechol, respectively (Figure 11). Thus, angular dioxygenation results in

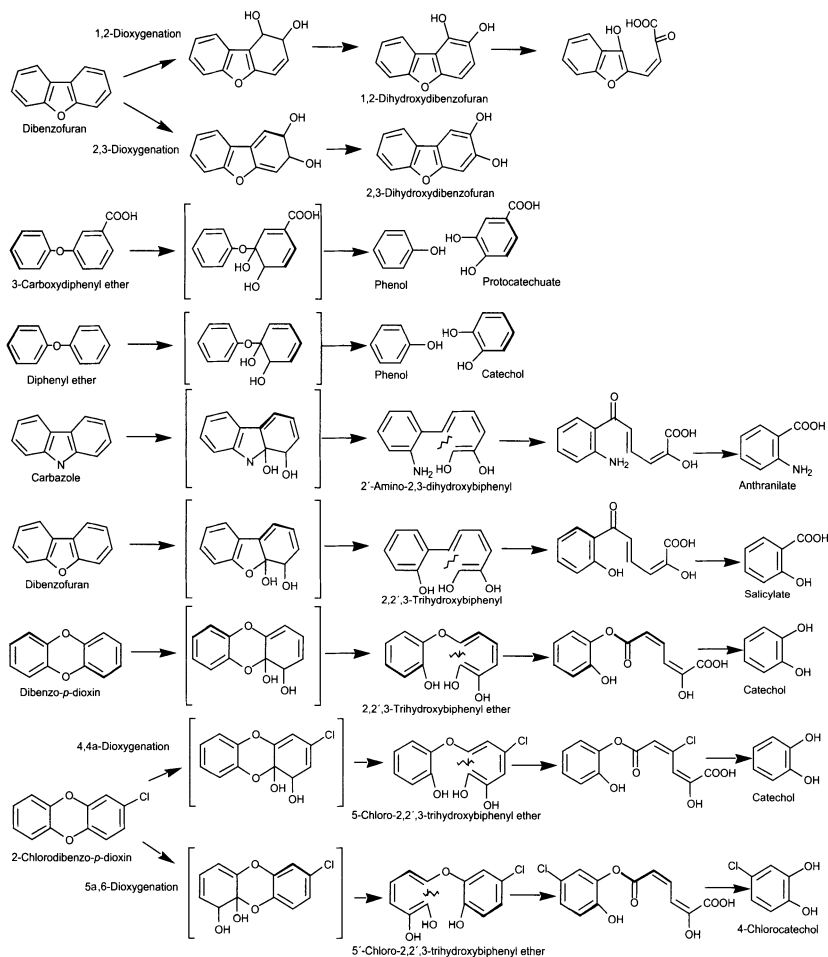


Figure 11. The metabolism of biarylethers and of carbazole. Top: Lateral dioxygenation of dibenzofuran⁴² gives rise to dead-end product. Bottom: The angular dioxygenation of 3-carboxydiphenyl ether⁷⁸, diphenyl ether³²¹, carbazole³¹¹, dibenzofuran⁸⁰ and dibenzo-*p*-dioxin³⁹¹ produces chemically unstable hemiacetal-like intermediates. Substituted biarylethers can be subject to different modes of angular dioxygenation^{112, 387}.

the cleavage of the three-ring structure or diphenyl ether structure. 2'-Substituted 2,3-dihydroxybiphenyls formed by angular dioxygenation are further degraded by *meta*-cleavage and hydrolysis, as shown for the degradation of biphenyl, resulting in the formation of salicylate (from dibenzofuran)^{88, 352}, catechol (from dibenzo-*p*-dioxin)³⁹¹ or anthranilate (from carbazole)³¹¹, respectively (Figure 11). Thus, after angular dioxygenation, subsequent degradation pathways are homologous to the corresponding biphenyl degradation pathway. So far, only a few bacterial strains capable of catalyzing lateral dioxygenation, have been described, dominantly *Pseudomonas*, *Sphingomonas* and *Terrabacter* strains.

The degradation of carboxybiphenyl ethers (or phenoxybenzoates) has been analyzed on the genetic level in *P. pseudoalcaligenes* POB310. A dioxygenase of the phthalate family (as shown for 3-chlorobenzoate 4,5-dioxygenase, which carries out a very similar kind of reaction) was shown to catalyze the angular dioxygenation⁶¹ forming phenol and protocatechuate. Similar metabolic routes have been observed in two strains tentatively identified as *Pseudomonas* sp.^{367, 390}. The dioxygenase of strain POB310 transforms various halosubstituted and even dichlorosubstituted derivatives with high rates^{78, 116}. Chlorophenols accumulated as dead-end products, whereas protocatechuate was degraded via protocatechuate *meta*-cleavage. However, growth on chlorosubstituted ethers was not observed, even though one of the aromatic rings could theoretically be used as a carbon source. Mineralization of 3-(4-chlorophenoxy)-benzoate could be achieved after transfer of the dioxygenase genes into the 4-chlorophenol mineralizing strain B13¹¹⁶.

Like phenoxybenzoate dioxygenase, various carbazole dioxygenases comprise an oxygenase of the phthalate family of Rieske-type non-heme iron oxygenases, however, the electron transport system is composed of a reductase and a ferredoxin. Carbazole dioxygenase α -subunits of *Pseudomonas resinovorans* CA10³¹¹, *P. stutzeri* OM1²⁶¹, *Pseudomonas* sp. K23 and *Janthinobacterium* sp. J3 are nearly identical, and highly homologous α -subunits (approx. 60% of sequence identity on amino acid level) were observed in *Sphingomonas* sp. strain KA1¹¹¹ and *Sphingomonas* sp. GTN11¹⁶⁴. Completely different carbazole degradation genes, which are related closer to biphenyl catabolic genes were observed in *Sphingomonas* sp. strain CB3³³⁷. Like dibenzofuran dioxygenase α -subunits of *Terrabacter* sp. strain DBF63¹⁵⁸, *Rhodococcus* sp. strain YK2 (AB070456), *Terrabacter* sp. strain YK3¹⁴⁴ and *S. wittichii* RW1⁹, the respective α -subunits are more related to the toluene/biphenyl and naphthalene families of Rieske non-heme iron oxygenases. However, only the α -subunits of strain YK3 and DBF63 are highly similar (only 2 amino acid differed in a stretch of 359 deduced amino acids). To the current information, angular dioxygenases thus do not form a monophyletic group, but belong to four lineages inside the toluene/biphenyl and naphthalene families of Rieske non-heme iron oxygenases. Genes highly similar to these lineages have not been reported thus far in *Pseudomonas* strains,

even though some angular dioxygenase genes were reported to be localized on mobile elements¹⁴⁴. In contrast, carbazole dioxygenases of the phthalate family were frequently observed in *Pseudomonas* sp. and the gene products were shown to be capable of transforming dibenzofuran and dibenzo-*p*-dioxin^{111, 311}, as well as chlorinated derivatives^{111, 112}. All strains analyzed in this aspect so far, share the capability to transform 2-chlorodibenzofuran and 2-chlorodibenzo-*p*-dioxin^{93, 112, 387}, however, differences were observed both in regioselectivity of attack and substrate specificity for transformation of chlorinated derivatives (Figure 11). As an example, both the enzyme of strains DBF63 and CA10 catalyze exclusively a 5a,6-dioxygenation¹¹², whereas the RW1 enzyme catalyzes both 4,4a- and 5a,6-dioxygenation³⁸⁷. 2,7-dichlorodibenzodioxin is not transformed by the strain DBF63 enzyme¹¹², and only with negligible rate by the strain RW1 enzyme³⁸⁷, whereas it is a good substrate for the strain CA10 enzyme¹¹². However, from the few reports available so far, no general structure/function relationships can be deduced.

A problem encountered during the degradation of dibenzofuran and chlorinated derivatives is the high instability of the trihydroxybiphenyl ring-cleavage products^{117, 178}, which give rise to their cycloisomerization products. Thus, an enzymatic hydrolysis to give salicylate has to compete against a spontaneous reaction, and only a balanced flux through the pathway will give rise to (chloro)salicylates. As an example, only cyclization products were observed to be formed from 2,8-dichlorodibenzofuran by strain RW1³⁸⁷, whereas high amounts of 5-chlorosalicylate were produced by strain DBF63. Strain CA10 cells produced only slight amounts of chlorosalicylate even from 2-chlorodibenzofuran. Even though the CA10 dioxygenase obviously was of broader substrate specificity than the DBF63 enzyme, catechols were not found to be formed from chlorinated dibenzodioxins. It was proposed that chlorinated trihydroxybiphenyl ether metabolites were not efficiently degraded by the carbazole upper pathway enzyme. Thus, the carbazole enzymatic system seems to be appropriate for chlorodibenzofuran and chlorodibenzodioxin depletion, however, in contrast to dioxin/dibenzofuran metabolic pathway enzymes, it seems to be inappropriate for further metabolism of the formed trihydroxybiphenyl derivatives¹¹².

8. ANAEROBIC DEGRADATION OF HALOAROMATIC COMPOUNDS BY *PSEUDOMONAS*

Many chloroaromatics serve as carbon and energy source for aerobic bacteria. Diverse dechlorination mechanisms exist either pre- or post-ring cleavage, including hydrolytic, reductive and oxygenolytic mechanisms²⁹⁰. Several of these steps were found to be spontaneous in nature, that is, the

enzymes convert a chlorinated substrate to an unstable product, thereby eliminating the chlorine substituent. Other dechlorination reactions were catalyzed by dehalogenases. Overall much biochemical data concerning the aerobic degradation of chloroaromatic compounds are available.

In contrast, the biochemical mechanisms involved in the anoxic dechlorination of chloroaromatic compounds are largely unknown. It is known since more than one decade that chloroaromatics can function as an alternative electron acceptor in a type of anaerobic respiration^{227, 228}. Several anaerobic bacteria, most dominantly *Dehalococcoides*^{2, 38, 54} or *Desulfitobacteria*³¹⁰ have been identified as being able to reductively dehalogenate chlorinated benzoates, benzenes, biphenyls and dioxins and to couple this reaction to the synthesis of ATP via a chemiosmotic mechanism.

However, anaerobic degradation of chloroaromatics not necessarily needs to be coupled to dehalorespiration. Anoxic microbial degradation of chloroaromatics was shown to take place with various electron acceptors such as nitrate, ferric ion, sulfate or carbon dioxide. The capability of denitrification is spread among *Pseudomonas*, and various dehalogenating enzymes described above (such as the 4-chlorobenzoate dehalogenase system) do not require oxygen and thus can function under anaerobic conditions, such that the observation of *Pseudomonas* degrading chloroaromatics under anaerobic conditions would not be surprising. Actually, the establishment of the *Pseudomonas* sp. CBS3 derived 4-chlorobenzoate dehalogenase genes in *Thauera aromatica* T1, which is capable of degrading toluene and 4-hydroxybenzoate under denitrifying conditions, resulted in a derivative mineralizing 4-chlorobenzoate anaerobically⁵⁰.

Under denitrifying conditions, 2-fluoro- and 3-fluoro- as well as 3-chloro- and 4-chlorobenzoate can readily be degraded^{114, 376}. Several bacterial strains capable of growing at the expense of aromatic compounds under denitrifying conditions have been assigned to the genera *Azoarcus* and *Thauera*^{90, 120}, and those organisms were assumed to be important for anaerobic toluene degradation *in situ*²⁶⁹. Members of these genera were also reported to be capable of degrading chloro- and fluorobenzoates under denitrifying conditions^{314, 343, 344}. More detailed analyses showed, however, that the capability to degrade halobenzoates under anoxic conditions is spread among the proteobacteria, and various *Pseudomonas* strains most closely related to *P. stutzeri*^{345, 376} degrading 2-fluoro- and 4-fluoro- as well as 3-chlorobenzoate have been isolated. Evidently, *P. stutzeri* strains are of high importance for halobenzoate degradation under natural conditions. However, the mechanisms of halobenzoate degradation under denitrifying conditions have not been elucidated yet. Previous work³¹⁴ had suggested that 2-fluorobenzoate dehalogenation occurs from the CoA-derivative by a fortuitous reaction, and in *Rhodopseudomonas palustris*, 3-chlorobenzoate degradation via reductive dehalogenation of 3-chlorobenzoyl-CoA to benzoyl-CoA was proposed⁷². In general, the

enzymes involved in anaerobic degradation of haloaromatics by pseudomonads remain to be elucidated.

9. IMPORTANCE OF *PSEUDOMONAS* STRAINS FOR CHLOROAROMATIC DEGRADATION *IN SITU*

The frequent isolation of *Pseudomonas* strains capable of degrading chloroaromatics seems to indicate that they are of importance for degradation also under environmental conditions. However, various catabolic pathways for chloroaromatics are plasmid encoded, and gene transfer occurs under environmental conditions. Different plasmids and mobile elements, specifically involved in the degradation of chlorobenzoate, 2,4-D or chloroaniline have been examined for their transfer under natural conditions and transconjugants receiving the plasmids have been characterized. Catabolic plasmids involved in 2,4-D degradation usually encode the whole set of enzymes necessary for transformation of 2,4-D into 3-oxoadipate, and thus transconjugants should be capable of efficiently expressing those genes. Generally it seems that the type of species recovered might have been inherent to the environment that provided the selection pressure. Selection advantages might be capabilities to maintain in biofilms or nutrient scavenging capabilities. In a detailed study on two horizons from an agricultural soil inoculated with a *P. putida* harboring one of two distinct catabolic plasmids, pJP4⁶⁵ or pEMT1³⁶⁵, changes in the community due to proliferation of transconjugants were observed⁶³, and a detailed phylogenetic analysis of these transconjugants showed that the plasmids were preferentially acquired and expressed in soil by representatives of *Ralstonia* and *Burkholderia*. *Stenothrophomonas* transconjugants were observed when easily degradable carbon sources were added together with the plasmid-bearing donor strain¹⁰⁹, however, none of the 95 isolates could be identified as *Pseudomonas*. Similarly, *Burkholderia* and *Ralstonia* transconjugants, but no *Pseudomonas* could be isolated after bioaugmentation of pJP4 donor strains into a sandy loam soil²⁴⁶.

Other studies examined strain collections. Among 18 unique 2,4-D degraders, strains belonging to members of the α , β and γ subgroups of the proteobacteria were identified, with isolates mainly characterized as *Burkholderia*, *Ralstonia* and *Sphingomonas*, but *Pseudomonas* was not isolated²²¹. A similar strain collection was described by Vallaeys *et al.*³⁷¹. From accumulated evidence it thus seems that 2,4-D degradation among easily culturable organisms is more spread among *Ralstonia* and *Burkholderia* strains than among *Pseudomonas* strains. However, those studies depend on the environment

analyzed. Subsurface aquifers constitute environments that are physically, chemically and biologically very different from surface soils with reduced concentration and availability of oxygen, carbon and nutrients and lower bacterial densities¹⁰⁴. The continuous exposure of such aquifers to chlorinated phenoxy-acetic acid herbicides has been shown to result in changes in the community composition and an increased abundance of *Pseudomonas*⁶⁰. Even though only 1 out of 50 *Pseudomonas* had the capability to mineralize 2,4-D, an important role of *Pseudomonas* on 2,4-D metabolism *in situ* was suggested. It is also known that rhizosphere bacteria such as fluorescent *Pseudomonas* sp. are ecologically adapted to colonize and compete in the rhizosphere environment. Expanding the metabolic functions of such pseudomonads to degrade pollutants may prove to be a useful strategy for bioremediation³³.

Even though *Pseudomonas* sp. strain B13 was isolated as a host of the *clc* element, in inoculation experiments into natural ecosystems, the element, like *tfd* genes, ended up in strains belonging to the genus *Ralstonia* or related β -proteobacteria like *Comamonas*^{289, 347, 412}. This was assumed to indicate that the *clc* genes are most efficiently expressed in strains belonging to those genera rather than in fluorescent pseudomonads. However, additional factors, compared to 2,4-D degradation govern the acquisition and spread of a 3-chlorobenzoate-degrading phenotype in nature. 3-Chlorobenzoate is activated by a chromosomal encoded benzoate dioxygenase and dehydrogenase by organisms harboring chlorocatechol genes. Thus, properties of the chlorobenzoate dioxygenase system of possible recipient strains can be regarded as selectivity factor³¹. Actually, *Pseudomonas* strains were obviously frequent among 3-chlorobenzoate-degrading isolates harboring a chlorocatechol *ortho*-cleavage pathway^{182, 268}.

However, another level of complexity regarding the degradation of chlorobenzoates is the existence of diverse pathways capable of achieving mineralization (Figure 12). Degradation of chlorobenzoates can occur via chlorocatechol (*clc* pathway), via hydrolytic dehalogenation of 4-chlorobenzoate to give 4-hydroxybenzoate (*fc*b pathway)¹⁷⁰, via dioxygenolytic dehalogenation of 3-chlorobenzoate to give 5-chloroprotocatechuate²⁴⁰ and probably via a fourth pathway with gentisate as an intermediate (*gp*-pathway)^{181, 183}. Recent studies have indicated that bacteria isolated from hypoxic habitats appear to have adapted metabolic routes for the degradation of aromatic compounds such that accumulation of toxic intermediates is avoided, and respective toluene-degrading *Pseudomonas* strains¹⁸⁵ harbored catechol dioxygenases with improved affinity for oxygen. Similarly, the archetype of the gentisate pathway for chlorobenzoate degradation (*Alcaligenes* sp. strain L6) was enriched under oxygen-limiting conditions¹⁸³. Obviously, bacteria harboring last mentioned pathway possessed relatively low growth rates on 3-chlorobenzoate and benzoate along with relatively high substrate and oxygen affinities for these compounds¹⁸². This is in contrast

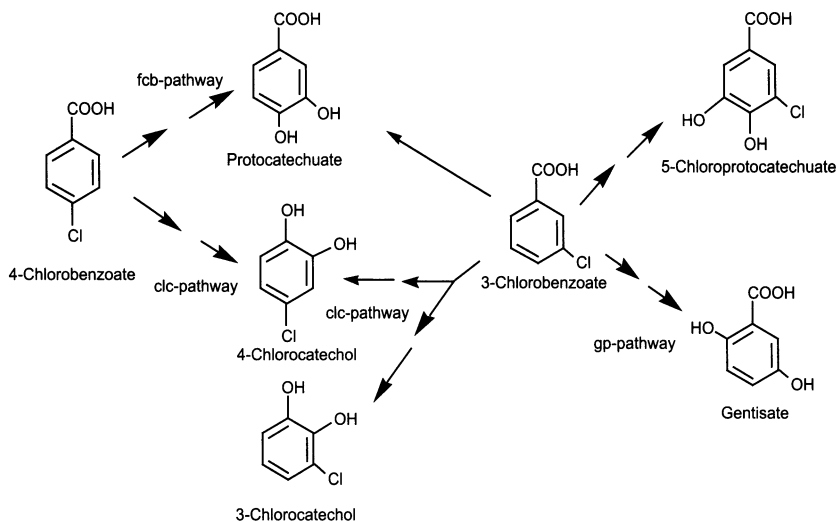


Figure 12. Diversity of pathways for 3-chloro- and 4-chlorobenzoate degradation. Pathways with chlorocatechol⁶⁷, (chloro)protocatechuate^{170, 240} and gentisate¹⁸³ have been reported.

with bacteria harboring the *clc* pathway, which seemed to be characterized by high maximum specific growth rates on the aromatic substrates and relatively high apparent half saturation constants. Thus, it was assumed that bacteria degrading chlorobenzoate via gentisate or protocatechuate might possibly be better adapted to conditions leading to growth at reduced rates such as low oxygen and low substrate concentrations. Evidently, the different pathways were observed to be spread in different taxa, and *Pseudomonas* sp. were observed to harbor *clc* genes, whereas *Bordetella* and *Alcaligenes* sp. were dominant among those of the *gp*-pathway¹⁸². Evidently, such clustering is due to the general genetic equipment of the organisms. However, gentisate dioxygenase genes were observed in different *P. aeruginosa* isolates¹³² and in the genome of *P. aeruginosa* PAO1³⁵¹, but not in the genome of *P. putida* KT2440. Whereas a degradative pathway of chlorobenzoate via gentisate has not been analyzed in great detail, the pathway via protocatechuate has been elucidated and the crucial chlorobenzoate dioxygenase was analyzed also on the genetic level. The host range of *cbaAB* genes for 3-chlorobenzoate 4,5-dioxygenase has been analyzed both in defined mating experiments as well as by analysis of transconjugants^{96, 241} formed under environmental conditions. Transconjugants were dominantly belonging to the β -proteobacteria, but also some *Pseudomonas* transconjugants were observed. However, those transconjugants dominantly formed 3-chlorocatechol from 3-chlorobenzoate, indicating an active benzoate 1,2-dioxygenase to be interfering with 3-chlorobenzoate 4,5-dioxygenase^{96, 241}. Moreover, 5-chloroprotocatechuate accumulated, indicating that protocatechuate 3,4-dioxygenase

cannot adequately deal with this metabolite. In contrast, mineralization of 3-chlorobenzoate was observed in organisms harboring a protocatechuate 4,5-dioxygenase, enzymes that can dehalogenate 5-chloroprotocatechuate¹⁶⁰. Thus, the host range of the *cbaAB* genes can be correlated with the distribution of the protocatechuate *meta*-ring-fission pathway²⁴¹. Hosts of the *fcB* genotype were, in contrast, mainly *Pseudomonas*²⁶⁸, which correlates with the capability of *Pseudomonas* to mineralize 4-hydroxybenzoate.

10. MICROBIAL COMMUNITIES

Although much information on metabolic pathways within individual organisms is available, little is known about the pathways operating in natural communities in which extensive sharing of nutritional resources is the rule. The most simple model systems are those involved in the degradation of bicyclic aromatics. Chlorinated biphenyl, dibenzofurans and dibenzo-*p*-dioxins, by most organisms isolated thus far, are not completely mineralized, but usually one aromatic ring is degraded, with the concomitant excretion of chlorobenzoates, chlorosalicylates or chlorocatechols^{140, 387, 391}. Defined mixed cultures have been described, which are capable^{8, 124, 392} of mineralizing chlorinated bicyclic compounds, and dominantly, *Pseudomonas* strains were introduced to achieve mineralization of the second ring.

Such a complete mineralization is desired, as substrate misrouting can result in the formation of toxic intermediates such as protoanemonin²⁷. A rapid decline in cell viability of different PCB-metabolizing organisms was observed in soil microcosms amended with 4-chlorobiphenyl¹²³. The toxic effect was due to protoanemonin formed from the transformation of 4-chlorobiphenyl by the natural microflora²⁶. Addition to soil microcosms of *Pseudomonas* strains able to reroute intermediary 4-chlorocatechol from the 3-oxoadipate pathway into the *meta*-cleavage pathway or able to mineralize 4-chlorocatechol by a modified *ortho*-cleavage pathway resulted in reversal of this toxic effect²⁶. A second group of intermediates of high toxicity are chlorocatechols³²⁶. If produced during metabolism, those compounds are highly toxic and can kill the producing organism²⁷¹, explaining why microorganisms grow on chloroaromatics, only when they harbor a balanced activity of chlorocatechol producing and consuming activities^{175, 271}.

Thus, under environmental conditions, communities will be important, where the different community members share their tasks. A respective model community consisting of four members, among them two *Pseudomonas* sp., and together capable of mineralizing a simple chloroaromatic, 4-chlorosalicylate, has recently been described²⁷⁰. Only one of the community members, an *Alcaligenes* strain, harbors a chlorocatechol pathway, but this organism only

represented 10% of the community and was supposed to degrade chlorocatechols excreted by the dominant community member *Pseudomonas* MT1. Both *Pseudomonas* MT1 and MT4 do not contain any known chloroaromatic degradation pathway. It seems thus, that metabolic and physiological weaknesses of primary degraders of xenobiotics maybe effectively compensated for by recruitment of other organisms with appropriate complementary physiology. However, such interactions will be overseen if only primary degraders are analyzed. Thus, the actual importance of *Pseudomonas* strains for degradation of chloroaromatics maybe higher than suggested just from analysis of the composition of primary degraders.

11. OUTLOOK

In this chapter we have been concerned with drawing together information from a variety of sources to illustrate the current state of knowledge on microbial degradation of chloroaromatic compounds. It was difficult to restrict this overview to *Pseudomonas* sp., as the actors in the processes. Closely related organisms, often formerly misclassified *Pseudomonas* sp., but also Gram-positive organisms, specifically *Rhodococci*, are known to be able to grow with chloroaromatic compounds. Even though *Pseudomonas* sp. are without doubt important for chloroaromatic degradation, as *Pseudomonas* are found in large numbers in different natural environments (soil, freshwater, marine) as well as in association with plants and animals, the fact that various catabolic genes are located on mobile elements enables numerous environmental organisms to integrate such foreign DNA. New molecular ecology tools, such as the combination of fluorescent in situ hybridization (to identify single bacteria in communities) with microautoradiography¹⁹² or stable isotope probing (the incorporation of labeled substrate in taxonomically relevant molecules such as 16S rRNA to identify active populations)²¹¹ will in the next years significantly advance our understanding on degradation of chloroaromatic compounds under environmental conditions.

Also it becomes more and more evident, that bacteria in the environment fight against each other⁵⁵, work together (carbon sharing)^{85, 270} or even built their own houses (clay hutches)²⁰⁹, or cities (biofilms)^{340, 350}. Bacteria in the environment are thus not simply the addition of diverse species but interacting communities. Understanding chloroaromatic degradation in the environment and the impact of *Pseudomonas* thus necessitates the understanding of these complex community interactions. However, these are, without doubt, significantly shaped by metabolic interactions²⁴⁸, such that an understanding of enzyme mechanisms, substrate misrouting and metabolic flux is a prerequisite to understand community interactions. Thus, analysis of single organisms or

simple model communities is still necessary. This is underlined by the still accumulating knowledge on new enzymes⁵⁶, enzyme mechanisms³⁴¹ and pathways for chloroaromatic degradation²²⁹, specifically under anaerobic conditions³⁸. Nevertheless, it is clear, that various degradation processes escape our knowledge when using classical microbiological strategies. Analysis of the bacterial meta-genome (the genetic information of entire microbial communities) is still in its infancy, but a powerful tool for exploring soil microbial diversity^{207, 301} and new enzymes important for chloroaromatic degradation still await to be discovered.

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REFERENCES

1. Abril, M.-A., Michan, C., Timmis, K.N., and Ramos, J.L., 1989, Regulator and enzyme specificities of the TOL plasmid-encoded upper pathway for degradation of aromatic hydrocarbons and expansion of the substrate range of the pathway. *J. Bacteriol.*, 171:6782–6790.
2. Adrian, L., Szewzyk, U., Wecke, J., and Görisch, H., 2000, Bacterial dehalorespiration with chlorinated benzenes. *Nature*, 408:580–583.
3. Ahmad, D., Sylvestre, M., and Sondossi, M., 1991, Subcloning of *bph* genes from *Pseudomonas testosteroni* B-356 in *Pseudomonas putida* and *Escherichia coli*: Evidence for dehalogenation during initial attack on chlorobiphenyls. *Appl. Environ. Microbiol.*, 57:2880–2887.
4. Anzai, Y., Kim, H.S., Park, J.Y., Wakabayashi, H., and Oyaizu, H., 2000, Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. *Int. J. Syst. Evol. Microbiol.*, 50:1563–1159.
5. Aoki, K., Otsuka, R., Shinke, R., and Nishina, H., 1984, Rapid biodegradation of aniline by *Frateria* species ANA-18 and its aniline metabolism. *Agric. Biol. Chem.*, 48:865–872.
6. Arensdorf, J.J. and Focht, D.D., 1994, Formation of chlorocatechol *meta* cleavage products by a pseudomonad during metabolism of monochlorobiphenyls. *Appl. Environ. Microbiol.*, 60:2884–2889.
7. Arensdorf, J.J. and Focht, D.D., 1995, A *meta* cleavage pathway for 4-chlorobenzoate, an intermediate in the metabolism of 4-chlorobiphenyl by *Pseudomonas cepacia* P166. *Appl. Environ. Microbiol.*, 61:443–447.
8. Arfmann, H.A., Timmis, K.N., and Wittich, R.M., 1997, Mineralization of 4-chlorodibenzofuran by a consortium consisting of *Sphingomonas* sp. strain RW1 and *Burkholderia* sp. strain JWS. *Appl. Environ. Microbiol.*, 63:3458–3462.
9. Armengaud, J., Happe, B., and Timmis, K.N., 1998, Genetic analysis of dioxin dioxygenase of *Sphingomonas* sp. strain RW1: Catabolic genes dispersed on the genome. *J. Bacteriol.*, 180:3954–3966.
10. Armengaud, J., Timmis, K.N., and Wittich, R.M., 1999, A functional 4-hydroxysalicylate/hydroxyquinol degradative pathway gene cluster is linked to the initial dibenzo-*p*-dioxin pathway genes in *Sphingomonas* sp. strain RW1. *J. Bacteriol.*, 181:3452–3461.

11. Asturias, J.A. and Timmis, K.N., 1993, Three different 2,3-dihydroxybiphenyl-1,2-dioxygenase genes in the gram-positive polychlorobiphenyl-degrading bacterium *Rhodococcus globerulus* P6. *J. Bacteriol.*, 175:4631–4640.
12. Babbitt, P.C., Kenyon, G.L., Martin, B.M., Charest, H., Sylvestre, M., Scholten, J.D., Chang, K.-H., Liang, P.-H., and Dunaway-Mariano, D., 1992, Ancestry of the 4-chlorobenzoate dehalogenase: Analysis of amino acid sequence identities among families of acyl:adenyl ligases, enoyl-CoA hydratases/isomerases, and acyl-CoA thioesterases. *Biochemistry*, 31:5594–5604.
13. Bartels, F., Backhaus, S., Moore, E.R.B., Timmis, K.N., and Hofer, B., 1999, Occurrence and expression of glutathione-S-transferase-encoding *bphK* genes in *Burkholderia* sp. strain LB400 and other biphenyl-utilizing bacteria. *Microbiology*, 145:2821–2834.
14. Bartels, I., Knackmuss, H.-J., and Reineke, W., 1984, Suicide inactivation of catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2 by 3-halocatechols. *Appl. Environ. Microbiol.*, 47:500–505.
15. Bayly, R.C., Dagley, S., and Gibson, D.T., 1966, The metabolism of cresols by species of *Pseudomonas*. *Biochem. J.*, 101:293–301.
16. Beadle, C.A. and Smith, A.R.W., 1982, The purification and properties of 2,4-dichlorophenol hydroxylase from a strain of *Acinetobacter* species. *Eur. J. Biochem.*, 123:323–332.
17. Bedard, D.L. and Haberl, M.L., 1990, Influence of chlorine substitution pattern on the degradation of polychlorinated biphenyls by eight bacterial strains. *Microb. Ecol.*, 20:87–102.
18. Bedard, D.L., Haberl, M.L., May, R.J., and Brennan, M.J., 1987, Evidence for novel mechanisms of polychlorinated biphenyl metabolism in *Alcaligenes eutrophus* H850. *Appl. Environ. Microbiol.*, 53:1103–1112.
19. Beil, S., Happe, B., Timmis, K.N., and Pieper, D.H., 1997, Genetic and biochemical characterization of the broad-spectrum chlorobenzene dioxygenase from *Burkholderia* sp. strain PS12: Dechlorination of 1,2,4,5-tetrachlorobenzene. *Eur. J. Biochem.*, 247:190–199.
20. Beil, S., Mason, J.R., Timmis, K.N., and Pieper, D.H., 1998, Identification of chlorobenzene dioxygenase sequence elements involved in dechlorination of 1,2,4,5-tetrachlorobenzene. *J. Bacteriol.*, 180:5520–5528.
21. Beil, S., Timmis, K.N., and Pieper, D.H., 1999, Genetic and biochemical analyses of the *tec* operon suggest a route for evolution of chlorobenzene degradation genes. *J. Bacteriol.*, 181:341–346.
22. Benning, M.M., Wesenberg, G., Liu, R.Q., Taylor, K.L., Dunaway-Mariano, D., and Holden, H.M., 1998, The three-dimensional structure of 4-hydroxybenzoyl-CoA thioesterase from *Pseudomonas* sp. strain CBS-3. *J. Biol. Chem.*, 273:33572–33579.
23. Bertoni, G., Bolognese, F., Galli, E., and Barbieri, P., 1996, Cloning of the genes for and characterization of the early stages of toluene and *o*-xylene catabolism in *Pseudomonas stutzeri* OX1. *Appl. Environ. Microbiol.*, 62:3704–3711.
24. Bertoni, G., Martino, M., Galli, E., and Barbieri, P., 1998, Analysis of the gene cluster encoding toluene/*o*-xylene monooxygenase from *Pseudomonas stutzeri* OX1. *Appl. Environ. Microbiol.*, 64:3626–3632.
25. Bianchi, D., Bosetti, A., Cidaria, D., Bernardi, A., Gagliardi, I., and Amico, P.D., 1997, Oxidation of polycyclic aromatic heterocycles by *Pseudomonas fluorescens* TTC1. *Appl. Microbiol. Biotechnol.*, 47:596–599.
26. Blasco, R., Mallavarapu, M., Wittich, R.M., Timmis, K.N., and Pieper, D.H., 1997, Evidence that formation of protoanemonin from metabolites of 4-chlorobiphenyl degradation negatively affects the survival of 4-chlorobiphenyl-cometabolizing microorganisms. *Appl. Environ. Microbiol.*, 63:427–434.
27. Blasco, R., Wittich, R.-M., Mallavarapu, M., Timmis, K.N., and Pieper, D.H., 1995, From xenobiotic to antibiotic. Formation of protoanemonin from 4-chlorocatechol by enzymes of the 3-oxoadipate pathway. *J. Biol. Chem.*, 270:29229–29235.

28. Bollag, J.-M., Briggs, G.G., Dawson, J.E., and Alexander, M., 1968, 2,4-D metabolism: Enzymatic degradation of chlorocatechol. *J. Agric. Food Chem.*, 16:829–833.
29. Bosch, R., Garcia-Valdes, E., and Moore, E.R.B., 1999, Genetic characterization and evolutionary implications of a chromosomally encoded naphthalene-degradation upper pathway from *Pseudomonas stutzeri* AN10. *Gene*, 236:149–157.
30. Bosch, R., Moore, E.R.B., Garcia-Valdes, E., and Pieper, D.H., 1999, NahW, a novel, inducible salicylate hydroxylase involved in mineralization of naphthalene by *Pseudomonas stutzeri* AN10. *J. Bacteriol.*, 181:2315–2322.
31. Bott, T.L. and Kaplan, L.A., 2002, Autecological properties of 3-chlorobenzoate-degrading bacteria and their population dynamics when introduced into sediments. *Microb. Ecol.*, 43:199–216.
32. Boyle, A.W., Silvén, C.J., Hassett, J.P., Nakas, J.P., and Tanenbaum, S.W., 1992, Bacterial PCB biodegradation. *Biodegradation*, 3:285–298.
33. Brazil, G.M., Kenefick, L., Callanan, M., Haro, A., de Lorenzo, V., Dowling, D.N., and O'Gara, F., 1995, Construction of a rhizosphere pseudomonad with potential to degrade polychlorinated biphenyls and detection of *bph* gene expression in the rhizosphere. *Appl. Environ. Microbiol.*, 61:1946–1952.
34. Brenner, V., Hernandez, B.S., and Focht, D.D., 1993, Variation in chlorobenzoate catabolism by *Pseudomonas putida* P111 as a consequence of genetic alterations. *Appl. Environ. Microbiol.*, 59:2790–2794.
35. Brinkmann, U. and Reineke, W., 1992, Degradation of chlorotoluenes by in vivo constructed hybrid strains: Problems of enzyme specificity, induction and prevention of *meta*-pathway. *FEMS Microbiol. Lett.*, 96:81–88.
36. Broderick, J.B. and O'Halloran, T.V., 1991, Overproduction, purification, and characterization of chlorocatechol dioxygenase, a non-heme iron dioxygenase with broad substrate tolerance. *Biochemistry*, 30:7349–7358.
37. Brunsbach, F.R. and Reineke, W., 1993, Degradation of chloroanilines in soil slurry by specialized organisms. *Appl. Microbiol. Biotechnol.*, 40:402–407.
38. Bunge, M., Adrian, L., Kraus, A., Opel, M., Lorenz, W.G., Andreesen, J.R., Görsch, H., and Lechner, U., 2003, Reductive dehalogenation of chlorinated dioxins by an anaerobic bacterium. *Nature*, 421:357–360.
39. Byrne, A.M., Kukor, J.J., and Olsen, R.H., 1995, Sequence analysis of the gene cluster encoding toluene-3-monooxygenase from *Pseudomonas pickettii* PKO1. *Gene*, 154:65–70.
40. Cai, M. and Xun, L.Y., 2002, Organization and regulation of pentachlorophenol-degrading genes in *Sphingobium chlorophenolicum* ATCC 39723. *J. Bacteriol.*, 184:4672–4680.
41. Cane, P.A. and Williams, P.A., 1982, The plasmid-coded metabolism of naphthalene and 2-methylnaphthalene in *Pseudomonas* strains: Phenotypic changes correlated with structural modification of the plasmid pWW60-1. *J. Gen. Microbiol.*, 128:2281–2290.
42. Cerniglia, C., Morgan, J., and Gibson, D.T., 1979, Bacterial and fungal oxidation of dibenzofuran. *Biochem. J.*, 180:175–185.
43. Chae, J., Kim, Y., Kim, Y.C., Zylstra, G.J., and Kim, C.K., 2000, Genetic structure and functional implication of the *fc*b gene cluster for hydrolytic dechlorination of 4-chlorobenzoate from *Pseudomonas* sp. DJ-12. *Gene*, 258:109–116.
44. Chang, K.H., Liang, P.H., Beck, W., Scholten, J.D., and Dunaway-Mariano, D., 1992, Isolation and characterization of the three polypeptide components of 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. strain CBS-3. *Biochemistry*, 31:5605–5610.
45. Chapman, P.J., 1979, Degradation mechanisms. In A.W. Bourquin and P.H. Pritchard (eds), *Microbial degradation of pollutants in marine environments*. EPA-600/9-79-012 Environmental Protection Agency, Gulf Breeze, FL, USA.
46. Chatterjee, D.K. and Chakrabarty, A.M., 1983, Genetic homology between independently isolated chlorobenzoate-degradative plasmids. *J. Bacteriol.*, 153:532–534.

47. Chatterjee, D.K., Kellogg, S.T., Hamada, S., and Chakrabarty, A.M., 1981, Plasmid specifying total degradation of 3-chlorobenzoate by a modified *ortho* pathway. *J. Bacteriol.*, 146:639–646.
48. Chatterjee, D.K., Kellogg, S.T., Watkins, D.R., and Chakrabarty, A.M., 1981, Plasmids in the biodegradation of chlorinated aromatic compounds. In S.B. Levy, R.C. Clowes, and E.L. Koenig (eds), *Molecular Biology, Pathogenicity, and Ecology of Bacterial Plasmids*, pp. 519–528. Plenum Press, New York.
49. Coco, W.M., Rothmel, R.K., Henikoff, S., and Chakrabarty, A.M., 1993, Nucleotide sequence and initial functional characterization of the *clcR* gene encoding a LysR family activator of the *clcABD* chlorocatechol operon in *Pseudomonas putida*. *J. Bacteriol.*, 175:417–427.
50. Coschigano, P.W., Häggblom, M.M., and Young, L.Y., 1994, Metabolism of both 4-chlorobenzoate and toluene under denitrifying conditions by a constructed bacterial strain. *Appl. Environ. Microbiol.*, 60:989–995.
51. Cowles, C.E., Nichols, N.N., and Harwood, C.S., 2000, BenR, a XylS homologue, regulates three different pathways of aromatic acid degradation in *Pseudomonas putida*. *J. Bacteriol.*, 182:6339–6346.
52. Crawford, R.L. and Mohn, W.W., 1985, Microbiological removal of pentachlorophenol from soil using a *Flavobacterium*. *Enzyme Microb. Technol.*, 7:617–620.
53. Crooks, G.P., Xu, L., Barkley, R.M., and Copley, S.D., 1995, Exploration of possible mechanisms for 4-chlorobenzoyl CoA dehalogenase: Evidence for an aryl-enzyme intermediate. *J. Am. Chem. Soc.*, 117:10791–10798.
54. Cutter, L.A., Watts, J.E.M., Sowers, K.R., and May, H.D., 2001, Identification of a microorganism that links its growth to the reductive dechlorination of 2,3,5,6-chlorobiphenyl. *Environ. Microbiol.*, 3:699–709.
55. Czarán, T.L., Hoekstra, R.F., and Pagie, L., 2002, Chemical warfare between microbes promotes biodiversity. *Proc. Natl. Acad. Sci. USA*, 99:786–790.
56. Dai, M.H., Rogers, J.B., Warner, J.R., and Copley, S.D., 2003, A previously unrecognized step in pentachlorophenol degradation in *Sphingobium chlorophenolicum* is catalyzed by tetrachlorobenzoquinone reductase (PcpD). *J. Bacteriol.*, 185:302–310.
57. Dai, S., Vaillancourt, F.H., Maroufi, H., Drouin, N.M., Neau, D.B., Snieckus, V., Nolin, J.T., and Eltis, L.D., 2002, Identification and analysis of a bottleneck in PCB degradation. *Nature Struct. Biol.*, 9:934–939.
58. Daubaras, D.L., Saido, K., and Chakrabarty, A.M., 1996, Purification of hydroxyquinol 1,2-dioxygenase and maleylacetate reductase: The lower pathway of 2,4,5-trichlorophenoxyacetic acid metabolism by *Burkholderia cepacia* AC1100. *Appl. Environ. Microbiol.*, 62:4276–4279.
59. de Bont, J.A.M., Vorage, M.A.J.W., Hartmans, S., and van den Tweel, W.J.J., 1986, Microbial degradation of 1,3-dichlorobenzene. *Appl. Environ. Microbiol.*, 52:677–680.
60. De Liphay, J.R., Tuxen, N., Johnsen, K., Hansen, L., Albrechtsen, H.J., Bjerg, P.L., and Aamand, J., 2003, In situ exposure to low herbicide concentrations affects microbial population composition and catabolic gene frequency in an aerobic shallow aquifer. *Appl. Environ. Microbiol.*, 69:461–467.
61. Dehmelt, U., Engesser, K.-H., Timmis, K.N., and Dwyer, D.F., 1995, Cloning, nucleotide sequence, and expression of the gene encoding a novel dioxygenase involved in metabolism of carboxy-diphenyl ethers in *Pseudomonas pseudoalcaligenes* POB310. *Arch. Microbiol.*, 163:35–41.
62. Dejonghe, W., Goris, J., Dierickx, A., de Dobbeleer, V., Crul, K., de Vos, P., Verstraete, W., and Top, E.M., 2002, Diversity of 3-chloroaniline and 3,4-dichloroaniline degrading bacteria isolated from three different soils and involvement of their plasmids in chloroaniline degradation. *FEMS Microbiol. Ecol.*, 42:315–325.
63. Dejonghe, W., Goris, J., El Fantroussi, S., Hofte, M., de Vos, P., Verstraete, W., and Top, E.M., 2000, Effect of dissemination of 2,4-dichlorophenoxyacetic acid (2,4-D) degradation

- plasmids on 2,4-D degradation and on bacterial community structure in two different soil horizons. *Appl. Environ. Microbiol.*, 66:3297–3304.
64. Don, R.H. and Pemberton, J.M., 1985, Genetic and physical map of the 2,4-dichlorophenoxyacetic acid-degradative plasmid pJP4. *J. Bacteriol.*, 161:466–468.
 65. Don, R.H. and Pemberton, J.M., 1981, Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. *J. Bacteriol.*, 145:681–686.
 66. Dong, J., Carey, P.R., Wei, Y.S., Luo, L.S., Lu, X.F., Liu, R.Q., and Dunaway-Mariano, D., 2002, Raman evidence for Meisenheimer complex formation in the hydrolysis reactions of 4-fluorobenzoyl- and 4-nitrobenzoyl-coenzyme A catalyzed by 4-chlorobenzoyl-coenzyme A dehalogenase. *Biochemistry*, 41:7453–7463.
 67. Dorn, E., Hellwig, M., Reineke, W., and Knackmuss, H.-J., 1974, Isolation and characterization of a 3-chlorobenzoate degrading pseudomonad. *Arch. Microbiol.*, 99:61–70.
 68. Dorn, E. and Knackmuss, H.-J., 1978, Chemical structure and biodegradability of halogenated aromatic compounds. Substituent effects on 1,2-dioxygenation of catechol. *Biochem. J.*, 174:85–94.
 69. Dorn, E. and Knackmuss, H.-J., 1978, Chemical structure and biodegradability of halogenated aromatic compounds. Two catechol 1,2-dioxygenases from a 3-chlorobenzoate-grown pseudomonad. *Biochem. J.*, 174:73–84.
 70. Ederer, M.M., Crawford, R.L., Herwig, R.P., and Orser, C.S., 1997, PCP degradation is mediated by closely related strains of the genus *Sphingomonas*. *Mol. Ecol.*, 6:39–49.
 71. Edgehill, R.U. and Finn, R.K., 1982, Isolation, characterization and growth kinetics of bacteria metabolizing pentachlorophenol. *Appl. Microbiol. Biotechnol.*, 16:179–184.
 72. Eglund, P.G., Gibson, J., and Harwood, C.S., 2001, Reductive, coenzyme A-mediated pathway for 3-chlorobenzoate degradation in the phototrophic bacterium *Rhodospseudomonas palustris*. *Appl. Environ. Microbiol.*, 67:1396–1399.
 73. Elsner, A., Löffler, F., Miyashita, K., Müller, R., and Lingens, F., 1991, Resolution of 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. strain CBS3 into three components. *Appl. Environ. Microbiol.*, 57:324–326.
 74. Eltis, L.D. and Bolin, J.T., 1996, Evolutionary relationships among extradiol dioxygenases. *J. Bacteriol.*, 178:5930–5937.
 75. Engelberts, K., Schmidt, E., and Reineke, W., 1989, Degradation of *o*-toluate by *Pseudomonas* sp. strain WR401. *FEMS Microbiol. Lett.*, 59:35–38.
 76. Engesser, K.-H., Schmidt, E., and Knackmuss, H.-J., 1980, Adaptation of *Alcaligenes eutrophus* B9 and *Pseudomonas* sp. B13 to 2-fluorobenzoate as growth substrate. *Appl. Environ. Microbiol.*, 39:68–73.
 77. Engesser, K.H., Auling, G., Busse, J., and Knackmuss, H.-J., 1990, 3-Fluorobenzoate enriched bacterial strain FLB 300 degrades benzoate and all three isomeric monofluorobenzoates. *Arch. Microbiol.*, 153:193–199.
 78. Engesser, K.H., Fietz, W., Fischer, P., Schulte, P., and Knackmuss, H.-J., 1990, Dioxygenolytic cleavage of aryl ether bonds: 1,2-dihydroxy-1,2-dihydroxy-4-carboxybenzophenone as evidence for initial 1,2-dioxygenation in 3- and 4-carboxy biphenyl ether degradation. *FEMS Microbiol. Lett.*, 69:317–322.
 79. Engesser, K.H. and Schulte, P., 1989, Degradation of 2-bromo-, 2-chloro- and 2-fluorobenzoate by *Pseudomonas putida* CLB 250. *FEMS Microbiol. Lett.*, 60:143–148.
 80. Engesser, K.H., Strubel, V., Christoglou, K., Fischer, P., and Rast, H.G., 1989, Dioxygenolytic cleavage of aryl ether bonds: 1,10-dihydro-1,10-dihydroxyfluoren-9-one, a novel arene dihydrodiol as evidence for angular dioxygenation of dibenzofuran. *FEMS Microbiol. Lett.*, 65:205–210.
 81. Erickson, B.D. and Mondello, F.J., 1993, Enhanced biodegradation of polychlorinated biphenyls after site-directed mutagenesis of a biphenyl dioxygenase gene. *Appl. Environ. Microbiol.*, 59:3858–3862.

82. Erickson, B.D. and Mondello, F.J., 1992, Nucleotide sequencing and transcriptional mapping of the genes encoding biphenyl dioxygenase, a multicomponent polychlorinated-biphenyl-degrading enzyme in *Pseudomonas* strain LB400. *J. Bacteriol.*, 174:2903–2912.
83. Eulberg, D., Kourbatova, E.M., Golovleva, L.A., and Schlömann, M., 1998, Evolutionary relationship between chlorocatechol catabolic enzymes from *Rhodococcus opacus* 1CP and their counterparts in proteobacteria: Sequence divergence and functional convergence. *J. Bacteriol.*, 180:1082–1094.
84. Evans, W.C., Smith, B.S.W., Moss, P., and Fernley, H.N., 1971, Bacterial metabolism of 4-chlorophenoxyacetate. *Biochem. J.*, 122:509–517.
85. Feigel, B.J. and Knackmuss, H.-J., 1993, Syntrophic interactions during degradation of 4-aminobenzenesulfonic acid by a two species bacterial culture. *Arch. Microbiol.*, 159: 124–130.
86. Fetzner, S., Müller, R., and Lingens, F., 1989, Degradation of 2-chlorobenzoate by *Pseudomonas cepacia* 2CBS. *Biol. Chem. Hoppe Seyler*, 370:1173–1182.
87. Fetzner, S., Müller, R., and Lingens, F., 1992, Purification and some properties of 2-halobenzoate 1,2-dioxygenase, a two component enzyme system from *Pseudomonas cepacia* 2CBS. *J. Bacteriol.*, 174:279–290.
88. Fortnagel, P., Harms, H., Wittich, R.-M., Krohn, S., Meyer, H., Sinnwell, V., Wilkes, H., and Francke, W., 1990, Metabolism of dibenzofuran by *Pseudomonas* sp. strain HH69 and the mixed culture HH27. *Appl. Environ. Microbiol.*, 56:1148–1156.
89. Frantz, B. and Chakrabarty, A.M., 1987, Organization and nucleotide sequence determination of a gene cluster involved in 3-chlorocatechol degradation. *Proc. Natl. Acad. Sci. USA*, 84:4460–4464.
90. Fries, M.R., Zhou, J., Chee-Sanford, J., and Tiedje, J.M., 1994, Isolation, characterization and distribution of denitrifying toluene degraders from a variety of habitats. *Appl. Environ. Microbiol.*, 60:2802–2810.
91. Fuenmayor, S.L., Wild, M., Boyes, A.L., and Williams, P.A., 1998, A gene cluster encoding steps in conversion of naphthalene to gentisate in *Pseudomonas* sp. strain U2. *J. Bacteriol.*, 180:2522–2530.
92. Fujii, T., Takeo, M., and Maeda, Y., 1997, Plasmid-encoded genes specifying aniline oxidation from *Acinetobacter* sp. strain YAA. *Microbiology*, 143:93–99.
93. Fukuda, K., Nagata, S., and Taniguchi, H., 2002, Isolation and characterization of dibenzofuran-degrading bacteria. *FEMS Microbiol. Lett.*, 208:179–185.
94. Fukuda, M., Yasukochi, Y., Kikuchi, Y., Nagata, Y., Kimbara, K., Horiuchi, H., Takagi, M., and Yano, K., 1994, Identification of the *bphA* and *bphB* genes of *Pseudomonas* sp. strain KKS102 involved in degradation of biphenyl and polychlorinated biphenyls. *Biochem. Biophys. Res. Commun.*, 202:850–856.
95. Fukumori, F. and Saint, C.P., 2001, Nucleotide sequences and regulational analysis of genes involved in conversion of aniline to catechol in *Pseudomonas putida* UCC22(pTDN1). *J. Bacteriol.*, 179:399–408.
96. Fulthorpe, R.R. and Wyndham, R.C., 1991, Transfer and expression of the catabolic plasmid pBRC60 in wild bacterial recipients in a freshwater ecosystem. *Appl. Environ. Microbiol.*, 57:1546–1553.
97. Furukawa, K., 2000, Engineering dioxygenases for efficient degradation of environmental pollutants. *Curr. Opin. Biotechnol.*, 11:244–249.
98. Furukawa, K., Hirose, J., Suyama, A., Zaiki, T., and Hayashida, S., 1993, Gene components responsible for discrete substrate specificity in the metabolism of biphenyl (*bph* operon) and toluene (*tod* operon). *J. Bacteriol.*, 175:5224–5232.
99. Furukawa, K. and Miyazaki, T., 1986, Cloning of a gene cluster encoding biphenyl and chlorobiphenyl degradation in *Pseudomonas pseudoalcaligenes*. *J. Bacteriol.*, 166:392–398.

100. Furukawa, K., Tomizuka, N., and Kamibayashi, A., 1979, Effect of chlorine substitution on the bacterial metabolism of various polychlorinated biphenyls. *Appl. Environ. Microbiol.*, 38:301–310.
101. Futamata, H., Harayama, S., and Watanabe, K., 2001, Group-specific monitoring of phenol hydroxylase genes for a functional assessment of phenol-stimulated trichloroethylene bioremediation. *Appl. Environ. Microbiol.*, 67:4671–4677.
102. Gartemann, K. and Eichenlaub, R., 2001, Isolation and characterization of IS1409, an insertion element of 4-chlorobenzoate-degrading *Arthrobacter* sp. strain TM1, and development of a system for transposon mutagenesis. *J. Bacteriol.*, 183:3729.
103. Gaunt, J.K. and Evans, W.C., 1971, Metabolism of 4-chloro-2-methylphenoxyacetate by a soil pseudomonad. *Biochem. J.*, 122:533–542.
104. Ghiorse, W.C. and Wilson, J.T., 1988, Microbial ecology of the terrestrial subsurface. *Adv. Appl. Microbiol.*, 33:107–172.
105. Gibson, D.T., Hensley, M., Yoshioka, H., and Mabry, T.J., 1970, Formation of (+)-*cis*-2,3-dihydroxy-1-methylcyclohexa-4,6-diene from toluene by *Pseudomonas putida*. *Biochemistry*, 9:1626–1630.
106. Gibson, D.T. and Parales, R.E., 2000, Aromatic hydrocarbon dioxygenases in environmental biotechnology. *Curr. Opin. Biotechnol.*, 11:236–243.
107. Göbel, M., Kassel-Cati, K., Schmidt, E., and Reineke, W., 2002, Degradation of aromatics and chloroaromatics by *Pseudomonas* sp. strain B13: Cloning, characterization, and analysis of sequences encoding 3-oxoadipate: succinyl-coenzyme A (CoA) transferase and 3-oxoadipyl-CoA thiolase. *J. Bacteriol.*, 184:216–223.
108. Goldman, P., Milne, G.W.A., and Pignataro, M.T., 1967, Fluorine containing metabolites formed from 2-fluorobenzoic acid by *Pseudomonas* species. *Arch. Biochem. Biophys.*, 118:178–184.
109. Goris, J., Dejonghe, W., Falsen, E., de Clerck, E., Geeraerts, B., Willems, A., Top, E.M., Vandamme, P., and de Vos, P., 2002, Diversity of transconjugants that acquired plasmid pJP4 or pEMT1 after inoculation of a donor strain in the A- and B-horizon of an agricultural soil and description of *Burkholderia hospita* sp. nov. and *Burkholderia terricola* sp. nov. *Syst. Appl. Microbiol.*, 25:340–352.
110. Haak, B., Fetzner, S., and Lingens, F., 1995, Cloning, nucleotide sequence, and expression of the plasmid-encoded genes for the two-component 2-halobenzoate 1,2-dioxygenase from *Pseudomonas cepacia* 2CBS. *J. Bacteriol.*, 177:667–675.
111. Habe, H., Ashikawa, Y., Saiki, Y., Yoshida, T., Nojiri, H., and Omori, T., 2002, *Sphingomonas* sp. strain KA1, carrying a carbazole dioxygenase gene homologue, degrades chlorinated dibenzo-*p*-dioxins in soil. *FEMS Microbiol. Lett.*, 211:43–49.
112. Habe, H., Chung, J.S., Lee, J.H., Kasuga, K., Yoshida, T., Nojiri, H., and Omori, T., 2001, Degradation of chlorinated dibenzofurans and dibenzo-*p*-dioxins by two types of bacteria having angular dioxygenases with different features. *Appl. Environ. Microbiol.*, 67:3610–3617.
113. Haddock, J.D., Horton, J.R., and Gibson, D.T., 1995, Dihydroxylation and dechlorination of chlorinated biphenyls by purified biphenyl 2,3-dioxygenase from *Pseudomonas* sp. strain LB400. *J. Bacteriol.*, 177:20–26.
114. Häggblom, M.M., Rivera, M.D., and Young, L.Y., 1996, Anaerobic degradation of halogenated benzoic acids coupled to denitrification observed in a variety of sediment and soil samples. *FEMS Microbiol. Lett.*, 144:213–219.
115. Haigler, B.E., Nishino, S.F., and Spain, J.C., 1988, Degradation of 1,2-dichlorobenzene by a *Pseudomonas* sp. *Appl. Environ. Microbiol.*, 54:294–301.
116. Halden, R.U., Peters, E.G., Halden, B.G., and Dwyer, D.F., 2000, Transformation of mono- and dichlorinated phenoxybenzoates by phenoxybenzoate-dioxygenase in *Pseudomonas pseudoalcaligenes* POB310 and a modified diarylether-metabolizing bacterium. *Biotechnol. Bioeng.*, 69:107–112.

117. Happe, B., Eltis, L., Poth, H., Hedderich, R., and Timmis, K.N., 1993, Characterization of 2,2',3-trihydroxybiphenyl dioxygenase, an extradiol dioxygenase from the dibenzofuran- and dibenzo-p-dioxin-degrading bacterium *Sphingomonas* sp. strain RW1. *J. Bacteriol.*, 175:7313–7320.
118. Harayama, S. and Kok, M., 1992, Functional and evolutionary relationships among diverse oxygenases. *Annu. Rev. Microbiol.*, 46:565–601.
119. Harayama, S., Rekik, M., Bairoch, A., Neidle, E.L., and Ornston, L.N., 1991, Potential DNA slippage structures acquired during evolutionary divergence of *Acinetobacter calcoaceticus* chromosomal *benABC* and *Pseudomonas putida* TOL pWW0 plasmid *xylXYZ*, genes encoding benzoate dioxygenases. *J. Bacteriol.*, 173:7540–7548.
120. Harms, G., Rabus, R., and Widdel, F., 1999, Anaerobic oxidation of the aromatic plant hydrocarbon p-cymene by newly isolated denitrifying bacteria. *Arch. Microbiol.*, 172:303–312.
121. Hartmann, J., Engelberts, K., Nordhaus, B., Schmidt, E., and Reineke, W., 1989, Degradation of 2-chlorobenzoate by in vivo constructed hybrid pseudomonads. *FEMS Microbiol. Lett.*, 61:17–22.
122. Harwood, C.S. and Parales, R.E., 1996, The β -ketoadipate pathway and the biology of self-identity. *Annu. Rev. Microbiol.*, 50:553–590.
123. Havel, J. and Reineke, W., 1992, Degradation of Aroclor 1221 and survival of strains in soil microcosms. *Appl. Microbiol. Biotechnol.*, 38:129–134.
124. Havel, J. and Reineke, W., 1991, Total degradation of various chlorobiphenyls by cocultures and in vivo constructed hybrid pseudomonads. *FEMS Microbiol. Lett.*, 78:163–170.
125. Hayase, N., Taira, K., and Furukawa, K., 1990, *Pseudomonas putida* KF715 *bphABCD* operon encoding biphenyl and polychlorinated biphenyl degradation: Cloning analysis, and expression in soil bacteria. *J. Bacteriol.*, 172:1160–1164.
126. Hein, P., Powlowski, J., Barriault, D., Hurtubise, Y., Ahmad, D., and Sylvestre, M., 1998, Biphenyl-associated *meta*-cleavage dioxygenases from *Comamonas testosteroni* B-356. *Can. J. Microbiol.*, 44:42–49.
127. Heiss, G., Stolz, A., Kuhm, A.E., Müller, C., Klein, J., Altenbuchner, J., and Knackmuss, H.-J., 1995, Characterization of a 2,3-dihydroxybiphenyl dioxygenase from the naphthalenesulfonate-degrading bacterium strain BN6. *J. Bacteriol.*, 177:5865–5871.
128. Hernandez, B.S., Higson, F.K., Kondrat, R., and Focht, D.D., 1991, Metabolism of and inhibition by chlorobenzoates in *Pseudomonas putida* P111. *Appl. Environ. Microbiol.*, 57: 3361–3366.
129. Hickey, W.J., Brenner, V., and Focht, D.D., 1992, Mineralization of 2-chloro- and 2,5-dichlorobiphenyl by *Pseudomonas* sp. strain UCR2. *FEMS Microbiol. Lett.*, 98:175–180.
130. Hickey, W.J. and Focht, D.D., 1990, Degradation of mono-, di-, and trihalogenated benzoic acids by *Pseudomonas aeruginosa* JB2. *Appl. Environ. Microbiol.*, 56:3842–3850.
131. Hickey, W.J. and Sabat, G., 2001, Integration of matrix-assisted laser desorption ionization-time of flight mass spectrometry and molecular cloning for the identification and functional characterization of mobile *ortho*-halobenzoate oxygenase genes in *Pseudomonas aeruginosa* strain JB2. *Appl. Environ. Microbiol.*, 67:5648–5655.
132. Hickey, W.J., Sabat, G., Yuroff, A.S., Arment, A.R., and Perez-Leshner, J., 2001, Cloning, nucleotide sequencing, and functional analysis of a novel, mobile cluster of biodegradation genes from *Pseudomonas aeruginosa* strain JB2. *Appl. Environ. Microbiol.*, 67:4603–4609.
133. Higson, F.K. and Focht, D.D., 1990, Degradation of 2-bromobenzoic acid by a strain of *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.*, 56:1615–1619.
134. Higson, F.K. and Focht, D.D., 1992, Utilization of 3-chloro-2-methylbenzoic acid by *Pseudomonas cepacia* MB2 through the *meta* fission pathway. *Appl. Environ. Microbiol.*, 58:2501–2504.

135. Hinteregger, C., Loidl, M., and Streichsbier, F., 1992, Characterization of isofunctional ring-cleavage enzymes in aniline and 3-chloroaniline degradation by *Pseudomonas acidovorans* CA28. *FEMS Microbiol. Lett.*, 97:261–266.
136. Hirose, J., Suyama, A., Hayashida, S., and Furukawa, K., 1994, Construction of hybrid biphenyl (*bph*) and toluene (*tod*) genes for functional analysis of aromatic ring dioxygenases. *Gene*, 138:27–33.
137. Hofer, B., Backhaus, S., and Timmis, K.N., 1994, The biphenyl/polychlorinated biphenyl-degradation locus (*bph*) of *Pseudomonas* sp. LB400 encodes four additional metabolic enzymes. *Gene*, 144:9–16.
138. Hollender, J., Dott, W., and Hopp, J., 1994, Regulation of chloro- and ethylphenol degradation in *Comamonas testosteroni* JH5. *Appl. Environ. Microbiol.*, 60:2330–2338.
139. Hollender, J., Hopp, J., and Dott, W., 1997, Degradation of 4-chlorophenol via the *meta* cleavage pathway by *Comamonas testosteroni* JH5. *Appl. Environ. Microbiol.*, 63:4567–4572.
140. Hong, H.B., Chang, Y.S., Nam, I.H., Fortnagel, P., and Schmidt, S., 2002, Biotransformation of 2,7-dichloro- and 1,2,3,4-tetrachlorodibenzo-*p*-dioxin by *Sphingomonas wittichii* RW1. *Appl. Environ. Microbiol.*, 68:2584–2588.
141. Hooper, S.W., Dockendorf, T.C., and Sayler, G.S., 1989, Characteristics and restriction analysis of the 4-chlorobiphenyl catabolic plasmid, pSS50. *Appl. Environ. Microbiol.*, 55:1286–1288.
142. Hrywna, Y., Tsoi, T.V., Maltseva, O.V., Quensen, J.F., and Tiedje, J.M., 1999, Construction and characterization of two recombinant bacteria that grow on *ortho*- and *para*-substituted chlorobiphenyls. *Appl. Environ. Microbiol.*, 65:2163–2169.
143. Hudlicky, T., Gonzalez, D., and Gibson, D.T., 1999, Enzymatic dihydroxylation of aromatics in enantioselective synthesis: Expanding asymmetric methodology. *Aldrich. Acta*, 32:35–62.
144. Iida, T., Mukouzaka, Y., Nakamura, K., and Kudo, T., 2002, Plasmid-borne genes code for an angular dioxygenase involved in dibenzofuran degradation by *Terrabacter* sp. strain YK3. *Appl. Environ. Microbiol.*, 68:3716–3723.
145. Janke, D. and Fritsche, W., 1979, Dechlorierung von 4-Chlorphenol nach extradioler Ringspaltung durch *Pseudomonas putida*. *Z. Allgem. Mikrobiol.*, 19:139–141.
146. Jeenes, D.J., Reineke, W., Knackmuss, H.-J., and Williams, P.A., 1982, TOL plasmid pWW0 in constructed halobenzoate-degrading *Pseudomonas* strains: Enzyme regulation and DNA structure. *J. Bacteriol.*, 150:180–187.
147. Jimenez, J.I., Minambres, B., Garcia, J.L., and Diaz, E., 2002, Genomic analysis of the aromatic catabolic pathways from *Pseudomonas putida* KT2440. *Environ. Microbiol.*, 4:824–841.
148. Johnson, G.R. and Olsen, R.H., 1997, Multiple pathways for toluene degradation in *Burkholderia* sp. strain JS150. *Appl. Environ. Microbiol.*, 63:4047–4052.
149. Johnson, G.R. and Olsen, R.H., 1995, Nucleotide sequence analysis of genes encoding a toluene/benzene-2-monooxygenase from *Pseudomonas* sp. strain JS150. *Appl. Environ. Microbiol.*, 61:3336–3346.
150. Jones, R., Pagmantidis, V., and Williams, P.A., 2000, *sal* genes determining the catabolism of salicylate esters are part of a superoperonic cluster of catabolic genes in *Acinetobacter* sp. strain ADP1. *J. Bacteriol.*, 182:2018–2025.
151. Kahng, H.Y., Malinverni, J.C., Majko, M.M., and Kukor, J.J., 2001, Genetic and functional analysis of the *tbc* operons for catabolism of alkyl- and chloroaromatic compounds in *Burkholderia* sp. strain JS150. *Appl. Environ. Microbiol.*, 67:4805–4816.
152. Kaphammer, B., Kukor, J.J., and Olsen, R.H., 1990, Regulation of *tfdCDEF* by *tdfR* of the 2,4-dichlorophenoxyacetic acid degradation plasmid JJP4. *J. Bacteriol.*, 172:2280–2286.
153. Karlson, U., Rojo, F., van Elsas, J.D., and Moore, E., 1996, Genetic and serological evidence for the recognition of four pentachlorophenol-degrading bacterial strains as a species of the genus *Sphingomonas*. *Syst. Appl. Microbiol.*, 18:539–548.

154. Kasberg, T., Seibert, V., Schlömann, M., and Reineke, W., 1997, Cloning, characterization, and sequence analysis of the *clcE* gene encoding the maleylacetate reductase of *Pseudomonas* sp. strain B13. *J. Bacteriol.*, 179:3801–3803.
155. Kaschabek, S.R., Kasberg, T., Müller, D., Mars, A.E., Janssen, D.B., and Reineke, W., 1998, Degradation of chloroaromatics: Purification and characterization of a novel type of chlorocatechol 2,3-dioxygenase of *Pseudomonas putida* GJ31. *J. Bacteriol.*, 180:296–302.
156. Kaschabek, S.R. and Reineke, W., 1992, Maleylacetate reductase of *Pseudomonas* sp. strain B13: Dechlorination of chloromaleylacetates, metabolites in the degradation of chloroaromatic compounds. *Arch. Microbiol.*, 158:412–417.
157. Kaschabek, S.R. and Reineke, W., 1995, Maleylacetate reductase of *Pseudomonas* sp. strain B13: Specificity of substrate conversion and halide elimination. *J. Bacteriol.*, 177:320–325.
158. Kasuga, K., Habe, H., Chung, J.S., Yoshida, T., Nojiri, H., Yamane, H., and Omori, T., 2001, Isolation and characterization of the genes encoding a novel oxygenase component of angular dioxygenase from the Gram-positive dibenzofuran-degrader *Terrabacter* sp. strain DBF63. *Biochem. Biophys. Res. Commun.*, 283:195–204.
159. Kaulmann, U., Kaschabek, S.R., and Schlömann, M., 2001, Mechanism of chloride elimination from 3-chloro- and 2,4-dichloro-*cis,cis*-muconate: New insight obtained from analysis of muconate cycloisomerase variant CatB-K169A. *J. Bacteriol.*, 183:4551–4561.
160. Kersten, P.J., Chapman, P.J., and Dagley, S., 1985, Enzymatic release of halogens or methanol from some substituted protocatechuic acids. *J. Bacteriol.*, 162:693–697.
161. Kersten, P.J., Dagley, S., Whittaker, J.W., Arciero, D., and Lipscomb, J.D., 1982, 2-Pyrone-4,6-dicarboxylic acid, a catabolite of gallic acids in *Pseudomonas* species. *J. Bacteriol.*, 152:1154–1162.
162. Kersters, K., Ludwig, W., Vancanneyt, M., de Vos, P., Gillis, M., and Schleifer, K.H., 1996, Recent change in the classification of the pseudomonads: An overview. *Syst. Appl. Microbiol.*, 19:465–477.
163. Khan, A. and Walia, S., 1989, Cloning of bacterial genes specifying degradation of 4-chlorobiphenyl from *Pseudomonas putida* OU83. *Appl. Environ. Microbiol.*, 55:798–805.
164. Kilbane, J.J., Daram, A., Abbasian, J., and Kayser, K.J., 2002, Isolation and characterization of *Sphingomonas* sp. GTIN11 capable of carbazole metabolism in petroleum. *Biochem. Biophys. Res. Commun.*, 297:242–248.
165. Kimura, N., Nishi, A., Goto, M., and Furukawa, K., 1997, Functional analyses of a variety of chimeric dioxygenases constructed from two biphenyl dioxygenases that are similar structurally but different functionally. *J. Bacteriol.*, 179:3936–3943.
166. Kitagawa, W., Miyauchi, K., Masai, E., and Fukuda, M., 2001, Cloning and characterization of benzoate catabolic genes in the gram-positive polychlorinated biphenyl degrader *Rhodococcus* sp. strain RHA1. *J. Bacteriol.*, 183:6598–6606.
167. Kitayama, A., Achioku, T., Yanagawa, T., Kanou, K., Kikuchi, M., Ueda, H., Suzuki, E., Nishimura, H., Nagamune, T., and Kawakami, Y., 1996, Cloning and characterization of extradiol aromatic ring-cleavage dioxygenases from *Pseudomonas aeruginosa* J1104. *J. Ferm. Bioeng.*, 82:217–223.
168. Kitayama, A., Suzuki, E., Kawakami, Y., and Nagamune, T., 1996, Gene organization and low regioselectivity in aromatic-ring hydroxylation of a benzene monooxygenase of *Pseudomonas aeruginosa* J1104. *J. Ferm. Bioeng.*, 82:421–425.
169. Klages, U. and Lingens, F., 1979, Degradation of 4-chlorobenzoic acid by a *Nocardia* species. *FEMS Microbiol. Lett.*, 6:201–203.
170. Klages, U. and Lingens, F., 1980, Degradation of 4-chlorobenzoic acid by a *Pseudomonas* sp. *Zbl. Bakteriell. Parasit. Infekt. Hyg. 1. Abt. Orig.*, C:215–223.
171. Klages, U., Markus, A., and Lingens, F., 1981, Degradation of 4-chlorophenylacetic acid by a *Pseudomonas* species. *J. Bacteriol.*, 146:64–68.

172. Klecka, G.M. and Gibson, D.T., 1981, Inhibition of catechol 2,3-dioxygenase from *Pseudomonas putida* by 3-chlorocatechol. *Appl. Environ. Microbiol.*, 41:1159–1165.
173. Klecka, G.M. and Gibson, D.T., 1981, Metabolism of dibenzo-*p*-dioxin and chlorinated dibenzo-*p*-dioxins by a *Beijerinckia* species. *Appl. Environ. Microbiol.*, 39:288–296.
174. Klecka, G.M. and Gibson, D.T., 1979, Metabolism of dibenzo(1,4)dioxin by a *Pseudomonas* species. *Biochem. J.*, 180:639–645.
175. Klemba, M., Jakobs, B., Wittich, R., and Pieper, D., 2000, Chromosomal integration of the *tcb* chlorocatechol degradation pathway genes as a means of expanding the growth substrate range of bacteria to include haloaromatics. *J. Bacteriol.*, 182:3255–3261.
176. Knackmuss, H.-J. and Hellwig, M., 1978, Utilization and cooxidation of chlorinated phenols by *Pseudomonas* sp. B13. *Arch. Microbiol.*, 117:1–7.
177. Kobayashi, K., Katayama-Hirayama, K., and Tobita, S., 1997, Hydrolytic dehalogenation of 4-chlorobenzoic acid by an *Acinetobacter* sp. *J. Gen. Appl. Microbiol.*, 43:105–108.
178. Kohler, H.-P.E., Schmid, A., and van der Maarel, M., 1993, Metabolism of 2,2'-dihydroxybiphenyl by *Pseudomonas* sp. strain HBP1: Production and consumption of 2,2',3-trihydroxybiphenyl. *J. Bacteriol.*, 175:1621–1628.
179. Koiv, V., Marits, R., and Heinaru, A., 1996, Sequence analysis of the 2,4-dichlorophenol hydroxylase gene *tfdB* and 3,5-dichlorocatechol 1,2-dioxygenase gene *tfdC* of 2,4-dichlorophenoxyacetic acid degrading plasmid pEST4011. *Gene*, 174:293–297.
180. Kozlovsky, S.A., Zaitsev, G.M., Kunc, F., Gabriel, J., and Boronin, A.M., 1993, Degradation of 2-chlorobenzoic and 2,5-dichlorobenzoic acids in pure culture by *Pseudomonas stutzeri*. *Folia Microbiol.*, 38:371–375.
181. Krooneman, J., Moore, E.R.B., van Velzen, J.C.L., Prins, R.A., Forney, L.J., and Gottschal, J.C., 1998, Competition for oxygen and 3-chlorobenzoate between two aerobic bacteria using different degradation pathways. *FEMS Microbiol. Ecol.*, 26:171–179.
182. Krooneman, J., Sliemers, A.O., Gomes, T.M.P., Forney, L.J., and Gottschal, J.C., 2000, Characterization of 3-chlorobenzoate degrading aerobic bacteria isolated under various environmental conditions. *FEMS Microbiol. Ecol.*, 32:53–59.
183. Krooneman, J., Wieringa, E.B.A., Moore, E.R.B., Gerritse, J., Prins, R.A., and Gottschal, J.C., 1996, Isolation of *Alcaligenes* sp. strain L6 at low oxygen concentrations and degradation of 3-chlorobenzoate via a pathway not involving (chloro)catechols. *Appl. Environ. Microbiol.*, 62:2427–2434.
184. Kuhm, A.E., Schlömann, M., Knackmuss, H.-J., and Pieper, D.H., 1990, Purification and characterization of dichloromuconate cycloisomerase from *Alcaligenes eutrophus* JMP134. *Biochem. J.*, 266:877–883.
185. Kukor, J.J. and Olsen, R.H., 1996, Catechol 2,3-dioxygenases functional in oxygen-limited (hypoxic) environments. *Appl. Environ. Microbiol.*, 62:1728–1740.
186. Kukor, J.J. and Olsen, R.H., 1990, Complete nucleotide sequence of *tbuD*, the gene encoding phenol/cresol hydroxylase from *Pseudomonas pickettii* PKO1, and functional analysis of the encoded enzyme. *J. Bacteriol.*, 174:6518–6526.
187. Kukor, J.J. and Olsen, R.H., 1990, Molecular cloning, characterization, and regulation of a *Pseudomonas pickettii* PKO1 gene encoding phenol hydroxylase and expression of the gene in *Pseudomonas aeruginosa* PAO1c. *J. Bacteriol.*, 172:4624–4630.
188. Latorre, J., Reineke, W., and Knackmuss, H.-J., 1984, Microbial metabolism of chloroanilines: Enhanced evolution by natural genetic exchange. *Arch. Microbiol.*, 140:159–165.
189. Latus, M., Seitz, H.-G., Eberspächer, J., and Lingens, F., 1995, Purification and characterization of hydroquinol 1,2-dioxygenase from *Azotobacter* sp. strain GP1. *Appl. Environ. Microbiol.*, 61:2453–2460.
190. Lee, J., Min, K.R., Kim, Y.-C., Kim, C.-K., Lim, J.-Y., Yoon, H., Min, K.-H., Lee, K.-S., and Kim, Y., 1995, Cloning of salicylate hydroxylase gene and catechol 2,3-dioxygenase gene

- and sequencing of an intergenic sequence between the two genes of *Pseudomonas putida* KF715. *Biochem. Biophys. Res. Commun.*, 211:382–388.
191. Lee, J.Y. and Xun, L.Y., 1997, Purification and characterization of 2,6-dichloro-p-hydroquinone chlorohydrolase from *Flavobacterium* sp. strain ATCC 39723. *J. Bacteriol.*, 179:1521–1524.
 192. Lee, N., Nielsen, P.H., Andreasen, K.H., Juretschko, S., Nielsen, J.L., Schleifer, K.H., and Wagner, M., 1999, Combination of fluorescent in situ hybridization and microautoradiography—a new tool for structure-function analyses in microbial ecology. *Appl. Environ. Microbiol.*, 65:1289–1297.
 193. Lee, S.G., Yoon, B.D., Park, Y.H., and Oh, H.M., 1998, Isolation of a novel pentachlorophenol-degrading bacterium, *Pseudomonas* sp. Bu34. *J. Appl. Microbiol.*, 85:1–8.
 194. Leesong, M., Henderson, B.S., Gillig, J.R., Schwab, J.M., and Smith, J.L., 1996, Structure of a dehydratase-isomerase from the bacterial pathway for biosynthesis of unsaturated fatty acids: Two catalytic activities in one active site. *Structure*, 4:253–264.
 195. Lehrbach, P.R., Zeyer, J., Reineke, W., Knackmuss, H.-J., and Timmis, K.N., 1984, Enzyme recruitment in vitro: Use of cloned genes to extend the range of haloaromatics degraded by *Pseudomonas* sp. strain B13. *J. Bacteriol.*, 158:1025–1032.
 196. Leung, K.T., Campbell, S., Gan, Y.D., White, D.C., Lee, H., and Trevors, J.T., 1999, The role of the *Sphingomonas* species UG30 pentachlorophenol-4-monooxygenase in p-nitrophenol degradation. *FEMS Microbiol. Lett.*, 173:247–253.
 197. Leveau, J.H.J., König, F., Fuchslin, H., Werlen, C., and van der Meer, J.R., 1999, Dynamics of multigene expression during catabolic adaptation of *Ralstonia eutropha* JMP134 (pJP4) to the herbicide 2,4-dichlorophenoxyacetate. *Mol. Microbiol.*, 33:396–406.
 198. Leveau, J.H.J. and van der Meer, J.R., 1996, The *tfdR* gene product can successfully take over the role of the insertion element-inactivated TfdT protein as a transcriptional activator of the *tfdCDEF* gene cluster, which encodes chlorocatechol degradation in *Ralstonia eutropha* JMP134(pJP4). *J. Bacteriol.*, 178:6824–6832.
 199. Liang, P.-H., Yang, G., and Dunaway-Mariano, D., 1993, Specificity of 4-chlorobenzoyl coenzyme A dehalogenase catalyzed dehalogenation of a halogenated aromatics. *Biochemistry*, 32:12245–12250.
 200. Liu, R.-Q., Liang, P.-H., Scholten, J., and Dunaway-Mariano, D., 1995, Transient state kinetic analysis of the chemical intermediates formed in the enzymatic dehalogenation of 4-chlorobenzoyl coenzyme A. *J. Am. Chem. Soc.*, 117:5003–5004.
 201. Liu, S., Ogawa, N., and Miyashita, K., 2001, The chlorocatechol degradative genes, *tfdT-CDEF*, of *Burkholderia* sp. strain NK8 are involved in chlorobenzoate degradation and induced by chlorobenzoates and chlorocatechols. *Gene*, 268:207–214.
 202. Liu, T. and Chapman, P.J., 1984, Purification and properties of a plasmid-encoded 2,4-dichlorophenol hydroxylase. *FEBS Lett.*, 173:314–318.
 203. Löffler, F., Lingens, F., and Müller, R., 1995, Dehalogenation of 4-chlorobenzoate. Characterisation of 4-chlorobenzoyl-coenzyme A dehalogenase from *Pseudomonas* sp. CBS3. *Biodegradation*, 6:203–212.
 204. Löffler, F., and Müller, R., 1991, Identification of 4-chlorobenzoyl-coenzyme A as intermediate in the dehalogenation catalyzed by 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. CBS3. *FEBS Lett.*, 290:224–226.
 205. Löffler, F., Müller, R., and Lingens, F., 1991, Dehalogenation of 4-chlorobenzoate by 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. CBS3: An ATP/coenzyme A dependent reaction. *Biochem. Biophys. Res. Commun.*, 176:1106–1111.
 206. Löffler, F., Müller, R., and Lingens, F., 1992, Purification and properties of 4-halobenzoate-coenzyme A ligase from *Pseudomonas* sp. CBS3. *Biol. Chem. Hoppe Seyler*, 373:1001–1007.

207. Lorenz, P., Liebeton, K., Niehaus, F., and Eck, J., 2002, Screening for novel enzymes for biocatalytic processes: Accessing the metagenome as a resource of novel functional sequence space. *Curr. Opin. Biotechnol.*, 13:572–577.
208. Louie, T.M., Webster, C.M., and Xun, L.Y., 2002, Genetic and biochemical characterization of a 2,4,6-trichlorophenol degradation pathway in *Ralstonia eutropha* JMP134. *J. Bacteriol.*, 184:3492–3500.
209. Lünsdorf, H., Erb, R.W., Abraham, W.R., and Timmis, K.N., 2000, “Clay hutches”: A novel interaction between bacteria and clay minerals. *Environ. Microbiol.*, 2:161–168.
210. Makdessi, K. and Lechner, U., 1997, Purification and characterization of 2,4-dichlorophenol hydroxylase isolated from a bacterium of the alpha-2 subgroup of the proteobacteria. *FEMS Microbiol. Lett.*, 157:95–101.
211. Manefield, M., Whiteley, A.S., Griffiths, R.I., and Bailey, M.J., 2002, RNA stable isotope probing, a novel means of linking microbial community function to phylogeny. *Appl. Environ. Microbiol.*, 68:5367–5373.
212. Männistö, M.K., Tiirila, M.A., Salkinoja-Salonen, M.S., Kulomaa, M.S., and Puhakka, J.A., 1999, Diversity of chlorophenol-degrading bacteria isolated from contaminated boreal groundwater. *Arch. Microbiol.*, 171:189–197.
213. Markus, A., Klages, U., Krauss, S., and Lingens, F., 1984, Oxidation and dehalogenation of 4-chlorophenylacetate by a two-component enzyme system from *Pseudomonas* sp. strain CBS3. *J. Bacteriol.*, 160:618–621.
214. Markus, A., Krekel, D., and Lingens, F., 1986, Purification and some properties of component A of the 4-chlorophenylacetate 3,4-dioxygenase from *Pseudomonas* species strain CBS. *J. Biol. Chem.*, 261:12883–12888.
215. Mars, A.E., Kasberg, T., Kaschabek, S.R., van Agteren, M.H., Janssen, D.B., and Reineke, W., 1997, Microbial degradation of chloroaromatics: Use of the *meta*-cleavage pathway for mineralization of chlorobenzene. *J. Bacteriol.*, 179:4530–4537.
216. Mars, A.E., Kingma, J., Kaschabek, S.R., Reineke, W., and Janssen, D.B., 1999, Conversion of 3-chlorocatechol by various catechol 2,3-dioxygenases and sequence analysis of the chlorocatechol dioxygenase region of *Pseudomonas putida* GJ31. *J. Bacteriol.*, 181:1309–1318.
217. Masai, E., Shinohara, S., Hara, H., Nishikawa, S., Katayama, Y., and Fukuda, M., 1999, Genetic and biochemical characterization of a 2-pyrone-4,6-dicarboxylic acid hydrolase involved in the protocatechuate 4,5-cleavage pathway of *Sphingomonas paucimobilis* SYK-6. *J. Bacteriol.*, 181:55–62.
218. McClure, N.C. and Venables, W.A., 1987, pTDN1, a catabolic plasmid involved in aromatic amine metabolism in *Pseudomonas putida* mt-2. *J. Gen. Microbiol.*, 133:2073–2077.
219. McCullar, M.V., Brenner, V., Adams, R.H., and Focht, D.D., 1994, Construction of a novel polychlorinated biphenyl-degrading bacterium: Utilization of 3,4-dichlorobiphenyl by *Pseudomonas acidovorans* M3GY. *Appl. Environ. Microbiol.*, 60:3833–3839.
220. McFall, S.M., Parsek, M.R., and Chakrabarty, A.M., 1997, 2-Chloromuconate and ClcR-mediated activation of the *clcABD* operon: In vitro transcriptional and DNase I footprint analyses. *J. Bacteriol.*, 179:3655–3663.
221. McGowan, C., Fulthorpe, R., Wright, A., and Tiedje, J.M., 1998, Evidence for interspecies gene transfer in the evolution of 2,4-dichlorophenoxyacetic acid degraders. *Appl. Environ. Microbiol.*, 64:4089–4092.
222. McKay, D.B., Prucha, M., Reineke, W., Timmis, K.N., and Pieper, D.H., 2003, Substrate specificity and expression of three 2,3-dihydroxybiphenyl 1,2-dioxygenases from *Rhodococcus globerulus* strain P6. *J. Bacteriol.*, 185:2944–2951.
223. McKay, D.B., Seeger, M., Zielinski, M., Hofer, B., and Timmis, K.N., 1997, Heterologous expression of biphenyl dioxygenase-encoding genes from a gram-positive broad-spectrum polychlorinated biphenyl degrader and characterization of chlorobiphenyl oxidation by the gene products. *J. Bacteriol.*, 179:1924–1930.

224. Milne, G.W.A., Goldman, P., and Holtzman, J.L., 1968, The metabolism of 2-fluorobenzoic acid. II. Studies with $^{18}\text{O}_2$. *J. Biol. Chem.*, 243:5374–5376.
225. Mitchell, K.H., Studts, J.M., and Fox, B.G., 2002, Combined participation of hydroxylase active site residues and effector protein binding in a *para* to *ortho* modulation of toluene 4-monooxygenase regiospecificity. *Biochemistry*, 41:3176–3188.
226. Miyauchi, K., Adachi, Y., Nagata, Y., and Takagi, M., 1999, Cloning and sequencing of a novel *meta*-cleavage dioxygenase gene whose product is involved in degradation of γ -hexachlorocyclohexane in *Sphingomonas paucimobilis*. *J. Bacteriol.*, 181:6712–6719.
227. Mohn, W.W. and Tiedje, J.M., 1991, Evidence for chemiosmotic coupling of reductive dechlorination and ATP synthesis in *Desulfomonile tiedjei*. *Arch. Microbiol.*, 157:1–6.
228. Mohn, W.W. and Tiedje, J.M., 1990, Strain DCB-1 conserves energy for growth from reductive dechlorination coupled to formate oxidation. *Arch. Microbiol.*, 153:267–271.
229. Moiseeva, O.V., Solyanikova, I.P., Kaschabek, S.R., Groning, J., Thiel, M., Golovleva, L.A., and Schlömann, M., 2002, A new modified *ortho* cleavage pathway of 3-chlorocatechol degradation by *Rhodococcus opacus* 1CP: Genetic and biochemical evidence. *J. Bacteriol.*, 184:5282–5292.
230. Mokross, H., Schmidt, E., and Reineke, W., 1990, Degradation of 3-chlorobiphenyl by in vivo constructed hybrid pseudomonads. *FEMS Microbiol. Lett.*, 71:179–186.
231. Mondello, F.J., Turcich, M.P., Lobos, J.H., and Erickson, B.D., 1997, Identification and modification of biphenyl dioxygenase sequences that determine the specificity of polychlorinated biphenyl degradation. *Appl. Environ. Microbiol.*, 63:3096–3103.
232. Moore, E., Mau, M., Arnscheidt, A., Böttger, E., Hutson, R., Collins, M., van de Peer, Y., de Wachter, R., and Timmis, K.N., 1996, The determination and comparison of the 16S rRNA gene sequences of species of the genus *Pseudomonas* (sensu stricto) and estimation of the natural intragenetic relationship. *Syst. Appl. Microbiol.*, 19:478–492.
233. Müller, D., Schlömann, M., and Reineke, W., 1996, Maleylacetate reductases in chloroaromatic-degrading bacteria using the modified *ortho* pathway: Comparison of catalytic properties. *J. Bacteriol.*, 178:298–300.
234. Müller, R., Oltmanns, R.H., and Lingens, F., 1988, Enzymatic dehalogenation of 4-chlorobenzoate by extracts from *Arthrobacter* sp. SU DSM 20407. *Biol. Chem. Hoppe Seyler*, 369:567–571.
235. Müller, R., Thiele, J., Klages, U., and Lingens, F., 1984, Incorporation of [^{18}O] water into 4-hydroxybenzoic acid in the reaction of 4-chlorobenzoate dehalogenase from *Pseudomonas* spec CBS3. *Biochem. Biophys. Res. Commun.*, 124:669–674.
236. Müller, T.A., Werlen, C., Spain, J.C., and van der Meer, J.R., 2003, Evolution of a chlorobenzene degradative pathway among bacteria in a contaminated groundwater mediated by a genomic island in *Ralstonia*. *Environ. Microbiol.*, 5:163–173.
237. Murakami, S., Okuno, T., Matsumura, E., Takenaka, S., Shinke, R., and Aoki, K., 1999, Cloning of a gene encoding hydroxyquinol 1,2-dioxygenase that catalyzes both intradiol and extradiol ring cleavage of catechol. *Biosci. Biotechnol. Biochem.*, 63:859–865.
238. Murakami, S., Takemoto, J., Takashima, A., Shinke, R., and Aoki, K., 1998, Purification and characterization of two muconate cycloisomerase isozymes from aniline-assimilating *Frateriia* species ANA-18. *Biosci. Biotechnol. Biochem.*, 62:1129–1133.
239. Murray, K., Duggleby, C.J., Sala-Trepat, J.M., and Williams, P.A., 1972, The metabolism of benzoate and methylbenzoates via the *meta*-cleavage by *Pseudomonas arvilla* mt-2. *Eur. J. Biochem.*, 28:301–310.
240. Nakatsu, C. and Wyndham, R.C., 1993, Cloning and expression of the transposable chlorobenzoate-3,4-dioxygenase genes of *Alcaligenes* sp. BR60. *Appl. Environ. Microbiol.*, 59:3625–3633.
241. Nakatsu, C.H., Fulthorpe, R.R., Holland, B.A., Peel, M.C., and Wyndham, R.C., 1995, The phylogenetic distribution of a transposable dioxygenase from the Niagara river watershed. *Mol. Ecol.*, 4:593–603.

242. Nakatsu, C.H., Providenti, M., and Wyndham, R.C., 1997, The *cis*-diol dehydrogenase *cbaC* gene of Tn5271 is required for growth on 3-chlorobenzoate but not 3,4-dichlorobenzoate. *Gene*, 196:209–218.
243. Nakatsu, C.H., Straus, N.A., and Wyndham, R.C., 1995, The nucleotide sequence of the Tn5271 3-chlorobenzoate 3,4-dioxygenase genes (*cbaAB*) unites the class IA oxygenase in a single lineage. *Microbiology*, 141:485–495.
244. Nam, J.W., Nojiri, H., Yoshida, T., Habe, H., Yamane, H., and Omori, T., 2001, New classification system for oxygenase components involved in ring-hydroxylating oxygenations. *Biosci. Biotechnol. Biochem.*, 65:254–263.
245. Nelson, K.E., Weinel, C., Paulsen, I.T., Dodson, R.J., Hilbert, H., dos Santos, V., Fouts, D.E., Gill, S.R., Pop, M., Holmes, M., Brinkac, L., Beanan, M., DeBoy, R.T., Daugherty, S., Kolonay, J., Madupu, R., Nelson, W., White, O., Peterson, J., Khouri, H., Hance, I., Lee, P.C., Holtzapple, E., Scanlan, D., Tran, K., Moazzez, A., Utterback, T., Rizzo, M., Lee, K., Kosack, D., Moestl, D., Wedler, H., Lauber, J., Stjepandic, D., Hoheisel, J., Straetz, M., Heim, S., Kiewitz, C., Eisen, J., Timmis, K.N., Dusterhoft, A., Tümmler, B., and Fraser, C.M., 2002, Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ. Microbiol.*, 4:799–808.
246. Newby, D.T., Gentry, T., and Pepper, I.L., 2000, Comparison of 2,4-dichlorophenoxyacetic acid degradation and plasmid transfer in soil resulting from bioaugmentation with two different pJP4 donors. *Appl. Environ. Microbiol.*, 66:3399–3407.
247. Newman, L.M. and Wackett, L.P., 1995, Purification and characterization of toluene 2-monooxygenase from *Burkholderia cepacia* G4. *Biochemistry*, 34:14066–14076.
248. Nielsen, A.T., Tolker-Nielsen, T., Barken, K.B., and Molin, S., 2000, Role of commensal relationships on the spatial structure of a surface-attached microbial consortium. *Environ. Microbiol.*, 2:59–68.
249. Nishi, A., Tominaga, K., and Furukawa, K., 2000, A 90-kilobase conjugative chromosomal element coding for biphenyl and salicylate catabolism in *Pseudomonas putida* KF715. *J. Bacteriol.*, 182:1949–1955.
250. Noda, Y., Nishikawa, S., Shiozuka, K.-I., Kadokuda, H., Nakajima, H., Yoda, K., Katayama, Y., Morohoshi, N., Haraguchi, T., and Yamasaki, M., 1990, Molecular cloning of the protocatechuate 4,5-dioxygenase genes of *Pseudomonas paucimobilis*. *J. Bacteriol.*, 172:2704–2709.
251. Nohynek, L.J., Nurmiaho-Lassila, E.L., Suhonen, E.L., Busse, H.J., Mohammadi, M., Hantula, J., Rainey, F., and Salkinoja-Salonen, M.S., 1996, Description of chlorophenol-degrading *Pseudomonas* sp. strains KF1(T), KF3, and NKF1 as a new species of the genus *Sphingomonas*, *Sphingomonas subarctica* sp. nov. *Int. J. Syst. Bacteriol.*, 46:1042–1055.
252. Nurk, A., Kasak, L., and Kivisaar, M., 1991, Sequence of the gene (*pheA*) encoding phenol monooxygenase from *Pseudomonas* sp. EST1001: Expression in *Escherichia coli* and *Pseudomonas putida*. *Gene*, 102:13–18.
253. Ogawa, N., McFall, S.M., Klem, T.J., Miyashita, K., and Chakrabarty, A.M., 1999, Transcriptional activation of the chlorocatechol degradative genes of *Ralstonia eutropha* NH9. *J. Bacteriol.*, 181:6697–6705.
254. Ogawa, N. and Miyashita, K., 1999, The chlorocatechol-catabolic transposon Tn5707 of *Alcaligenes eutrophus* NH9, carrying a gene cluster highly homologous to that in the 1,2,4-trichlorobenzene-degrading bacterium *Pseudomonas* sp. strain P51, confers the ability to grow on 3-chlorobenzoate. *Appl. Environ. Microbiol.*, 65:724–731.
255. Ohtsubo, Y., Miyauchi, K., Kanda, K., Hatta, T., Kiyohara, H., Senda, T., Nagata, Y., Mitsui, Y., and Takagi, M., 1999, PcpA, which is involved in the degradation of pentachlorophenol in *Sphingomonas chlorophenolica* ATCC39723, is a novel type of ring-cleavage dioxygenase. *FEBS Lett.*, 459:395–398.
256. Olsen, R.H., Kukor, J.J., and Kaphammer, B., 1994, A novel toluene-3-monooxygenase pathway cloned from *Pseudomonas pickettii* PKO1. *J. Bacteriol.*, 176:3749–3756.

257. Oltmanns, R.H., Müller, R., Otto, M.K., and Lingens, F., 1989, Evidence for a new pathway in the bacterial degradation of 4-fluorobenzoate. *Appl. Environ. Microbiol.*, 55:2499–2504.
258. Oltmanns, R.H., Rast, H.G., and Reineke, W., 1988, Degradation of 1,4-dichlorobenzene by enriched and constructed bacteria. *Appl. Microbiol. Biotechnol.*, 28:609–616.
259. Orser, C.S., Dutton, J., Lange, C., Jablonski, P., Xun, L., and Hargis, M., 1993, Characterization of a *Flavobacterium* glutathione S-transferase gene involved in reductive dechlorination. *J. Bacteriol.*, 175:2640–2644.
260. Orser, C.S., Lange, C.C., Xun, L., Zahrt, T.C., and Schneider, B.J., 1993, Cloning, sequence analysis, and expression of the *Flavobacterium* pentachlorophenol-4-monooxygenase gene in *Escherichia coli*. *J. Bacteriol.*, 175:411–416.
261. Ouchiya, N., Miyachi, S., and Omori, T., 1998, Cloning and nucleotide sequence of carbazole catabolic genes from *Pseudomonas stutzeri* strain OM1, isolated from activated sludge. *J. Gen. Appl. Microbiol.*, 44:57–63.
262. Padilla, L., Matus, V., Zenteno, P., and Gonzalez, B., 2000, Degradation of 2,4,6-trichlorophenol via chlorohydroxyquinol in *Ralstonia eutropha* JMP134 and JMP222. *J. Basic Microbiol.*, 40:243–249.
263. Palleroni, N.J. and Bradbury, J.F., 1993, *Stenotrophomonas*, a new bacterial genus for *Xanthomonas maltophilia* (Hugh 1980) Swings *et al.*, 1983. *Int. J. Syst. Bacteriol.*, 43:606–609.
264. Palleroni, N.J., Kunisawa, R., Contopoulou, R., and Douderoff, M., 1973, Nucleic acid homologies in the genus *Pseudomonas*. *Int. J. Syst. Microbiol.*, 23:333–339.
265. Parales, J.V., Parales, R.E., Resnick, S.M., and Gibson, D.T., 1998, Enzyme specificity of 2-nitrotoluene 2,3-dioxygenase from *Pseudomonas* sp. strain JS42 is determined by the C-terminal region of the alpha subunit of the oxygenase component. *J. Bacteriol.*, 180:1194–1199.
266. Park, H.S. and Kim, H.S., 2000, Identification and characterization of the nitrobenzene catabolic plasmids pNB1 and pNB2 in *Pseudomonas putida* HS12. *J. Bacteriol.*, 182:573–580.
267. Pavlu, L., Vosahlova, J., Klierova, H., Prouza, M., Demnerova, K., and Brenner, V., 1999, Characterization of chlorobenzoate degraders isolated from polychlorinated biphenyl-contaminated soil and sediment in the Czech Republic. *J. Appl. Microbiol.*, 87:381–386.
268. Peel, M.C. and Wyndham, R.C., 1999, Selection of *clc*, *cba*, and *fc*b chlorobenzoate-catabolic genotypes from groundwater and surface waters adjacent to the Hyde Park, Niagara Falls, chemical landfill. *Appl. Environ. Microbiol.*, 65:1627–1635.
269. Pelz, O., Chatzinotas, A., Andersen, N., Bernasconi, S.M., Hesse, C., Abraham, W.R., and Zeyer, J., 2001, Use of isotopic and molecular techniques to link toluene degradation in denitrifying aquifer microcosms to specific microbial populations. *Arch. Microbiol.*, 175:270–281.
270. Pelz, O., Tesar, M., Wittich, R.M., Moore, E.R.B., Timmis, K.N., and Abraham, W.R., 1999, Towards elucidation of microbial community metabolic pathways: Unravelling the network of carbon sharing in a pollutant-degrading bacterial consortium by immunocapture and isotopic ratio mass spectrometry. *Environ. Microbiol.*, 1:167–174.
271. Perez-Pantoja, D., Ledger, T., Pieper, D.H., and Gonzalez, B., 2003, Efficient turnover of chlorocatechols is essential for growth of *Ralstonia eutropha* JMP134(pJP4) in 3-chlorobenzoic acid. *J. Bacteriol.*, 185:1534–1542.
272. Perkins, E.J., Gordon, M.P., Caceres, O., and Lurquin, P.F., 1990, Organization and sequence analysis of the 2,4-dichlorophenol hydroxylase and dichlorocatechol oxidative operons of plasmid pJP4. *J. Bacteriol.*, 172:2351–2359.
273. Pieper, D.H., Knackmuss, H.-J., and Timmis, K.N., 1993, Accumulation of 2-chloromucate during metabolism of 3-chlorobenzoate by *Alcaligenes eutrophus* JMP134. *Appl. Microbiol. Biotechnol.*, 39:563–567.
274. Pieper, D.H., Kuhm, A.E., Stadler-Fritzsche, K., Fischer, P., and Knackmuss, H.-J., 1991, Metabolization of 3,5-dichlorocatechol by *Alcaligenes eutrophus* JMP 134. *Arch. Microbiol.*, 156:218–222.

275. Pieper, D.H. and Reineke, W., 2000, Engineering bacteria for bioremediation. *Curr. Opin. Biotechnol.*, 11:262–270.
276. Pikus, J.D., Studts, J.M., McClay, K., Steffan, R.J., and Fox, B.G., 1997, Changes in the regiospecificity of aromatic hydroxylation produced by active site engineering in the diiron enzyme toluene 4-monooxygenase. *Biochemistry*, 36:9283–9289.
277. Pinyakong, O., Habe, H., Yoshida, T., Nojiri, H., and Omori, T., 2003, Identification of three novel salicylate 1-hydroxylases involved in the phenanthrene degradation by *Sphingobium* sp. strain P2. *Biochem. Biophys. Res. Commun.*, 301:350–357.
278. Plumeier, I., Perez-Pantoja, D., Heim, S., Gonzalez, B., and Pieper, D.H., 2002, Importance of different *tfd* genes for degradation of chloroaromatics by *Ralstonia eutropha* JMP134. *J. Bacteriol.*, 184:4054–4064.
279. Pollmann, K., Kaschabek, S., Wray, V., Reineke, W., and Pieper, D.H., 2002, Metabolism of dichloromethylcatechols as central intermediates in the degradation of dichlorotoluenes by *Ralstonia* sp. strain PS12. *J. Bacteriol.*, 184:5261–5274.
280. Pollmann, K., Wray, V., Hecht, H.J., and Pieper, D.H., 2003, Rational engineering of the regioselectivity of TecA tetrachlorobenzene dioxygenase for the transformation of chlorinated toluenes. *Microbiology*, 149:903–913.
281. Potrawfke, T., Armengaud, J., and Wittich, R.M., 2001, Chlorocatechols at positions 4 and 5 are substrates of the broad-spectrum chlorocatechol 1,2-dioxygenase of *Pseudomonas chlororaphis* RW71. *J. Bacteriol.*, 183:997–1011.
282. Powlowski, J., Sealy, J., Shingler, V., and Cadieux, E., 1997, On the role of DmpK, an auxiliary protein associated with multicomponent phenol hydroxylase from *Pseudomonas* sp. CF600. *J. Biol. Chem.*, 272:945–951.
283. Providenti, M.A. and Wyndham, R.C., 2001, Identification and functional characterization of CbaR, a MarR-like modulator of the *cbaABC*-encoded chlorobenzoate catabolism pathway. *Appl. Environ. Microbiol.*, 67:3530–3541.
284. Prucha, M., Peterseim, A., Timmis, K.N., and Pieper, D.H., 1996, Muconolactone isomerase of the 3-oxoadipate pathway catalyzes dechlorination of 5-chloro-substituted muconolactones. *Eur. J. Biochem.*, 237:350–356.
285. Prucha, M., Wray, V., and Pieper, D.H., 1996, Metabolism of 5-chlorosubstituted muconolactones. *Eur. J. Biochem.*, 237:357–366.
286. Radehaus, P. and Schmidt, S., 1992, Characterization of a new *Pseudomonas* sp. that mineralizes high concentrations of pentachlorophenol. *Appl. Environ. Microbiol.*, 58:2879–2885.
287. Ravatn, R., Studer, S., Springael, D., Zehnder, A.J.B., and van der Meer, J.R., 1998, Chromosomal integration, tandem amplification, and deamplification in *Pseudomonas putida* F1 of a 105-kilobase genetic element containing the chlorocatechol degradative genes from *Pseudomonas* sp. strain B13. *J. Bacteriol.*, 180:4360–4369.
288. Ravatn, R., Studer, S., Zehnder, A.J.B., and van der Meer, J.R., 1998, Int-B13, an unusual site-specific recombinase of the bacteriophage P4 integrase family, is responsible for chromosomal insertion of the 105-kilobase *clc* element of *Pseudomonas* sp. strain B13. *J. Bacteriol.*, 180:5505–5514.
289. Ravatn, R., Zehnder, A.J.B., and van der Meer, J.R., 1998, Low-frequency horizontal transfer of an element containing the chlorocatechol degradation genes from *Pseudomonas* sp. strain B13 to *Pseudomonas putida* F1 and to indigenous bacteria in laboratory-scale activated-sludge microcosms. *Appl. Environ. Microbiol.*, 64:2126–2132.
290. Reineke, W., 2001, Aerobic and anaerobic biodegradation potentials of microorganisms. In O. Hutzinger (ed.), *The Handbook of Environmental Chemistry*, vol. 2K, pp. 1–161. *The Natural Environment and Biogeochemical Cycles*. Springer Verlag, Berlin.
291. Reineke, W., 1998, Development of hybrid strains for the mineralization of chloroaromatics by patchwork assembly. *Annu. Rev. Microbiol.*, 52:287–331.

292. Reineke, W., Jeenes, D.J., Williams, P.A., and Knackmuss, H.-J., 1982, TOL plasmid pWW0 in constructed halobenzoate-degrading *Pseudomonas* strains: Prevention of *meta* pathway. *J. Bacteriol.*, 150:195–201.
293. Reineke, W. and Knackmuss, H.-J., 1978, Chemical structure and biodegradability of halogenated aromatic compounds. Substituent effects on 1,2-dioxygenation of benzoic acid. *Biochim. Biophys. Acta*, 532:412–423.
294. Reineke, W. and Knackmuss, H.-J., 1978, Chemical structure and biodegradability of halogenated aromatic compounds. Substituent effects on dehydrogenation of 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid. *Biochim. Biophys. Acta*, 542:424–429.
295. Reineke, W. and Knackmuss, H.-J., 1979, Construction of haloaromatics utilising bacteria. *Nature*, 277:385–386.
296. Resnick, S.M. and Chapman, P.J., 1994, Physiological properties and substrate specificity of a pentachlorophenol degrading *Pseudomonas* species. *Biodegradation*, 5:47–54.
297. Resnick, S.M. and Gibson, D.T., 1996, Regio- and stereospecific oxidation of fluorene, dibenzofuran, and dibenzothiophene by naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816-4. *Appl. Environ. Microbiol.*, 62:4073–4080.
298. Romanov, V. and Hausinger, R.P., 1994, *Pseudomonas aeruginosa* 142 uses a three-component *ortho*-halobenzoate 1,2-dioxygenase for the metabolism of 2,4-dichloro- and 2-chlorobenzoate. *J. Bacteriol.*, 176:3368–3374.
299. Romanov, V.P., Grechikina, G.M., Adanin, V.M., and Starovoitov, I.I., 1993, Oxidative dehalogenation of 2-chloro- and 2,4-dichlorobenzoates by *Pseudomonas aeruginosa*. *Microbiology*, 62:532–536.
300. Romine, M.F., Stillwell, L.C., Wong, K.K., Thurston, S.J., Sisk, E.C., Sensen, C., Gaasterland, T., Fredrickson, J.K., and Saffer, J.D., 1999, Complete sequence of a 184-kilobase catabolic plasmid from *Sphingomonas aromaticivorans* F199. *J. Bacteriol.*, 181:1585–1602.
301. Rondon, M.R., August, P.R., Bettermann, A.D., Brady, S.F., Grossman, T.H., Liles, M.R., Loiacono, K.A., Lynch, B.A., MacNeil, I.A., Minor, C., Tiong, C.L., Gilman, M., Osburne, M.S., Clardy, J., Handelsman, J., and Goodman, R.M., 2000, Cloning the soil metagenome: A strategy for accessing the genetic and functional diversity of uncultured microorganisms. *Appl. Environ. Microbiol.*, 66:2541–2547.
302. Rossello-Mora, R.A., Lalucat, J., and Garcia-Valdes, E., 1994, Comparative biochemical and genetic analysis of naphthalene degradation among *Pseudomonas stutzeri* strains. *Appl. Environ. Microbiol.*, 6:966–972.
303. Rubio, M.A., Engesser, K.-H., and Knackmuss, H.-J., 1986, Microbial metabolism of chlorosalicylates: Accelerated evolution by natural genetic exchange. *Arch. Microbiol.*, 145:116–122.
304. Ruisinger, S., Klages, U., and Lingens, F., 1976, Abbau der 4-Chlorbenzoesäure durch eine *Arthrobacter* species. *Arch. Microbiol.*, 110:253–256.
305. Saber, D.L. and Crawford, R.L., 1985, Isolation and characterization of *Flavobacterium* strains that degrade pentachlorophenol. *Appl. Environ. Microbiol.*, 50:1512–1518.
306. Saboo, V.M. and Gealt, M.A., 1998, Gene sequences of the *pcpB* gene of pentachlorophenol-degrading *Sphingomonas chlorophenolica* found in nondegrading bacteria. *Can. J. Microbiol.*, 44:667–675.
307. Saint, C.P., McClure, N.C., and Venables, W.A., 1990, Physical map of the aromatic amine and m-toluate catabolic plasmid pTDN1 in *Pseudomonas putida*: Location of a unique *meta*-cleavage pathway. *J. Gen. Microbiol.*, 136:615–625.
308. Sakai, M., Masai, E., Asami, H., Sugiyama, K., Kimbara, K., and Fukuda, M., 2002, Diversity of 2,3-dihydroxybiphenyl dioxygenase genes in a strong PCB degrader, *Rhodococcus* sp. strain RHA1. *J. Biosci. Bioeng.*, 93:421–427.
309. Sander, P., Wittich, R.-M., Fortnagel, P., Wilkes, H., and Francke, W., 1991, Degradation of 1,2,4-trichloro- and 1,2,4,5-tetrachlorobenzene by *Pseudomonas* strains. *Appl. Environ. Microbiol.*, 57:1430–1440.

310. Sanford, R.A., Cole, J.R., Löffler, F.E., and Tiedje, J.M., 1996, Characterization of *Desulfitobacterium chlororespirans* sp. nov., which grows by coupling the oxidation of lactate to the reductive dechlorination of 3-chloro-4-hydroxybenzoate. *Appl. Environ. Microbiol.*, 62:3800–3808.
311. Sato, S.I., Nam, J.W., Kasuga, K., Nojiri, H., Yamane, H., and Omori, T., 1997, Identification and characterization of genes encoding carbazole 1,9a-dioxygenase in *Pseudomonas* sp. strain CA10. *J. Bacteriol.*, 179:4850–4858.
312. Savard, P., Charest, H., Sylvestre, M., Shareck, F., Scholten, J.D., and Dunaway-Mariano, D., 1992, Expression of the 4-chlorobenzoate dehalogenase genes from *Pseudomonas* sp. CBS3 in *Escherichia coli* and identification of the gene translation products. *Can. J. Microbiol.*, 38:1074–1083.
313. Schell, U., Helin, S., Kajander, T., Schlömann, M., and Goldman, A., 1999, Structural basis for the activity of two muconate cycloisomerase variants toward substituted muconates. *Prot. Struct. Funct. Genet.*, 34:125–136.
314. Schennen, U., Braun, K., and Knackmuss, H.-J., 1985, Anaerobic degradation of 2-fluorobenzoate by benzoate-degrading, denitrifying bacteria. *J. Bacteriol.*, 161:321–325.
315. Schlömann, M., 1994, Evolution of chlorocatechol catabolic pathways. Conclusions to be drawn from comparisons of lactone hydrolases. *Biodegradation*, 5:301–321.
316. Schlömann, M., Fischer, P., Schmidt, E., and Knackmuss, H.-J., 1990, Enzymatic formation, stability, and spontaneous reactions of 4-fluoromuconolactone, a metabolite of the bacterial degradation of 4-fluorobenzoate. *J. Bacteriol.*, 172:5119–5129.
317. Schlömann, M., Schmidt, E., and Knackmuss, H.-J., 1990, Different types of dienelactone hydrolase in 4-fluorobenzoate-utilizing bacteria. *J. Bacteriol.*, 172:5112–5118.
318. Schmidt, E. and Knackmuss, H.-J., 1980, Chemical structure and biodegradability of halogenated aromatic compounds. Conversion of chlorinated muconic acids into maleoylacetic acid. *Biochem. J.*, 192:339–347.
319. Schmidt, E., Remberg, G., and Knackmuss, H.-J., 1980, Chemical structure and biodegradability of halogenated aromatic compounds. Halogenated muconic acids as intermediates. *Biochem. J.*, 192:331–337.
320. Schmidt, S. and Kirby, G.W., 2001, Dioxygenative cleavage of C-methylated hydroquinones and 2,6-dichlorohydroquinone by *Pseudomonas* sp. HH35. *Biochim. Biophys. Acta*, 1568:83–89.
321. Schmidt, S., Wittich, R.-M., Erdmann, D., Wilkes, H., Francke, W., and Fortnagel, P., 1992, Biodegradation of diphenyl ether and its monohalogenated derivatives by *Sphingomonas* sp. strain SS3. *Appl. Environ. Microbiol.*, 58:2744–2750.
322. Schmitz, A., Gartemann, K.-H., Fiedler, J., Grund, E., and Eichenlaub, R., 1992, Cloning and sequence analysis of genes for dehalogenation of 4-chlorobenzoate from *Arthrobacter* sp. strain SU. *Appl. Environ. Microbiol.*, 58:4068–4071.
323. Scholten, J.D., Chang, K.-H., Babbitt, P.C., Charest, H., Sylvestre, M., and Dunaway-Mariano, D., 1991, Novel enzymic hydrolytic dehalogenation of a chlorinated aromatic. *Science*, 253:182–185.
324. Schraa, G., Boone, M.L., Jetten, M.S.M., van Neerven, A.R.W., Colberg, P.J., and Zehnder, A.J.B., 1986, Degradation of 1,4-dichlorobenzene by *Alcaligenes* sp. strain A175. *Appl. Environ. Microbiol.*, 52:1374–1381.
325. Schreiber, A., Hellwig, M., Dorn, E., Reineke, W., and Knackmuss, H.-J., 1980, Critical reactions in fluorobenzoic acid degradation by *Pseudomonas* sp. B13. *Appl. Environ. Microbiol.*, 39:58–67.
326. Schweigert, N., Zehnder, A.J.B., and Eggen, R.I.L., 2001, Chemical properties of catechols and their molecular modes of toxic action in cells, from microorganisms to mammals. *Environ. Microbiol.*, 3:81–91.

327. Schweizer, D., Markus, A., Seez, M., Ruf, H., and Lingens, F., 1987, Purification and some properties of component B of the 4-chlorophenylacetate 3,4-dioxygenase from *Pseudomonas* species strain CBS 3. *J. Biol. Chem.*, 262:9340–9346.
328. Schwien, U. and Schmidt, E., 1982, Improved degradation of monochlorophenols by a constructed strain. *Appl. Environ. Microbiol.*, 44:33–39.
329. Seah, S.Y.K., Labbe, G., Kaschabek, S.R., Reifenrath, F., Reineke, W., and Eltis, L.D., 2001, Comparative specificities of two evolutionarily divergent hydrolases involved in microbial degradation of polychlorinated biphenyls. *J. Bacteriol.*, 183:1511–1516.
330. Seah, S.Y.K., Labbe, G., Nerdinger, S., Johnson, M.R., Snieckus, V., and Eltis, L.D., 2000, Identification of a serine hydrolase as a key determinant in the microbial degradation of polychlorinated biphenyls. *J. Biol. Chem.*, 275:15701–15708.
331. Seegal, B.C. and Holden, M., 1945, The antibiotic activity of extracts of Ranunculaceae. *Science*, 101:413–414.
332. Seeger, M., Timmis, K.N., and Hofer, B., 1997, Bacterial pathways for the degradation of polychlorinated biphenyls. *Marine Chem.*, 58:327–333.
333. Seeger, M., Timmis, K.N., and Hofer, B., 1995, Degradation of chlorobiphenyls catalyzed by the *bph*-encoded biphenyl-2,3-dioxygenase and biphenyl-2,3-dihydrodiol-2,3-dehydrogenase of *Pseudomonas* sp. LB400. *FEMS Microbiol. Lett.*, 133:259–264.
334. Seeger, M., Zielinski, M., Timmis, K.N., and Hofer, B., 1999, Regiospecificity of dioxygenation of di- to pentachlorobiphenyls and their degradation to chlorobenzoates by the *bph*-encoded catabolic pathway of *Burkholderia* sp. strain LB400. *Appl. Environ. Microbiol.*, 65:3614–3621.
335. Segers, P., Vancanneyt, M., Pot, B., Torck, U., Hoste, B., Dewettinck, D., Felsen, E., Kersters, K., and de Vos, P., 1994, Classification of *Pseudomonas diminuta* Leifson and Hugh 1954 and *Pseudomonas vesticularis* Büsing, Döll, and Freytag in *Brevundimonas* gen. nov. as *Brevundimonas diminuta* comb. nov. and *Brevundimonas vesticularis* comb. nov., respectively. *Int. J. Syst. Microbiol.*, 44:499–510.
336. Selifonov, S.A., Slep'en'kin, A.V., Adanin, V.M., Nefedova, M.Y., and Starovoitov, 1992, Oxidation of dibenzofuran by *Pseudomonas* harboring plasmids for naphthalene degradation. *Mikrobiologiya*, 60:67–71.
337. Shepherd, J.M. and Lloyd-Jones, G., 1998, Novel carbazole degradation genes of *Sphingomonas* CB3: Sequence analysis, transcription, and molecular ecology. *Biochem. Biophys. Res. Commun.*, 247:129–135.
338. Shields, M.S., Montgomery, S.O., Chapman, P.J., Cuskey, S.M., and Pritchard, P.H., 1989, Novel pathway of toluene catabolism in the trichloroethylene-degrading bacterium G4. *Appl. Environ. Microbiol.*, 55:1624–1629.
339. Shingler, V., Powlowski, J., and Marklund, U., 1992, Nucleotide sequence and functional analysis of the complete phenol/3,4-dimethylphenol catabolic pathway of *Pseudomonas* sp. strain CF600. *J. Bacteriol.*, 174:711–724.
340. Shirtliff, M.E., Mader, J.T., and Camper, A.K., 2002, Molecular interactions in biofilms. *Chem. Biol.*, 9:859–871.
341. Skiba, A., Hecht, V., and Pieper, D.H., 2002, Formation of protoanemonin from 2-chloro-*cis,cis*-muconate by the combined action of muconate cycloisomerase and muconolactone isomerase. *J. Bacteriol.*, 184:5402–5409.
342. Solyanikova, I.P., Malteva, O.V., Vollmer, M.D., Golovleva, L.A., and Schlömann, M., 1995, Characterization of muconate and chloromuconate cycloisomerase from *Rhodococcus erythropolis* ICP: Indications for functionally convergent evolution among bacterial cycloisomerases. *J. Bacteriol.*, 177:2821–2826.
343. Song, B., Kerkhof, L.J., and Häggblom, M.M., 2002, Characterization of bacterial consortia capable of degrading 4-chlorobenzoate and 4-bromobenzoate under denitrifying conditions. *FEMS Microbiol. Lett.*, 213:183–188.

344. Song, B., Palleroni, N., Kerkhof, L.J., and Häggblom, M.M., 2001, Characterization of halobenzoate-degrading, denitrifying *Azoarcus* and *Thauera* isolates and description of *Thauera chlorobenzoica* sp. nov. *Int. J. Syst. Evol. Microbiol.*, 51:589–602.
345. Song, B.K., Palleroni, N.J., and Häggblom, M.M., 2000, Isolation and characterization of diverse halobenzoate-degrading denitrifying bacteria from soils and sediments. *Appl. Environ. Microbiol.*, 66:3446–3453.
346. Springael, D., Kreps, S., and Mergeay, M., 1993, Identification of a catabolic transposon, Tn4371, carrying biphenyl and 4-chlorobiphenyl degradation genes in *Alcaligenes eutrophus* A5. *J. Bacteriol.*, 175:1674–1681.
347. Springael, D., Peys, K., Ryngaert, A., van Roy, S., Hooyberghs, L., Ravatn, R., Heyndrickx, M., van der Meer, J.R., Vandecasteele, C., Mergeay, M., and Diels, L., 2002, Community shifts in a seeded 3-chlorobenzoate degrading membrane biofilm reactor: Indications for involvement of in situ horizontal transfer of the *clc*-element from inoculum to contaminant bacteria. *Environ. Microbiol.*, 4:70–80.
348. Stanier, R.Y., Palleroni, N., and Doudoroff, M., 1966, The aerobic *Pseudomonas*: A taxonomic study. *J. Gen. Microbiol.*, 43:159–271.
349. Stanier, R.Y. and Ornston, L.N., 1973, The β -ketoadipate pathway. In A.H. Rose and D.W. Tempest (eds), *Advances in Microbial Physiology*, vol. 9, pp. 89–151. Academic press, London.
350. Stickler, D., 1999, Biofilms. *Curr. Opin. Microbiol.*, 2:270–275.
351. Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrenner, P., Hickey, M.J., Brinkman, F.S.L., Hufnagle, W.O., Kowalik, D.J., Lagrou, M., Garber, R.L., Goltry, L., Tolentino, E., Westbrook-Wadman, S., Yuan, Y., Brody, L.L., Coulter, S.N., Folger, K.R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G.K.S., Wu, Z., Paulsen, I.T., Reizer, J., Saier, M.H., Hancock, R.E.W., Lory, S., and Olson, M.V., 2000, Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature*, 406:959–964.
352. Strubel, V., Engesser, K.-H., Fischer, P., and Knackmuss, H.-J., 1991, 3-(2-Hydroxyphenyl) catechol as substrate for proximal *meta* ring cleavage in dibenzofuran degradation by *Brevibacterium* sp. strain DPO 1361. *J. Bacteriol.*, 173:1932–1937.
353. Suenaga, H., Nishi, A., Watanabe, T., Sakai, M., and Furukawa, K., 1999, Engineering a hybrid pseudomonad to acquire 3,4-dioxygenase activity for polychlorinated biphenyls. *J. Biosci. Bioeng.*, 87:430–435.
354. Suzuki, K., Ogawa, N., and Miyashita, K., 2001, Expression of 1,2-halobenzoate dioxygenase genes (*cbdSABC*) involved in the degradation of benzoate and 2-halobenzoate in *Burkholderia* sp. TH2. *Gene*, 262:137–145.
355. Taira, K., Hirose, J., Hayashida, S., and Furukawa, K., 1991, Analysis of *bph* operon from the polychlorinated biphenyl-degrading strain of *Pseudomonas pseudoalcaligenes* KF707. *J. Biol. Chem.*, 267:4844–4853.
356. Takeuchi, M., Hamana, K., and Hiraishi, A., 2001, Proposal of the genus *Sphingomonas* sensu stricto and three new genera, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*, on the basis of phylogenetic and chemotaxonomic analyses. *Int. J. Syst. Evol. Microbiol.*, 51:1405–1417.
357. Takizawa, N., Yokoyama, H., Yanagihara, K., Hatta, T., and Kiyohara, H., 1995, A locus of *Pseudomonas pickettii* DTP0602, *had*, that encodes 2,4,6-trichlorophenol-4-dechlorinase with hydroxylase activity, and hydroxylation of various chlorophenols by the enzyme. *J. Ferm. Bioeng.*, 80:318–326.
358. Tamaoka, J., Ha, D., and Komagata, K., 1987, Reclassification of *Pseudomonas acidovorans* den Dooren de Jong 1926 and *Pseudomonas testosteroni* Marcus and Talalay 1956 as *Comamonas acidovorans* com. nov. and *Comamonas testosteroni* comb. nov., with an emended description of the genus *Comamonas*. *Int. J. Syst. Microbiol.*, 37:52–59.

359. Tan, H.-M. and Cheong, C.-M., 1994, Substitution of the ISP α subunit of biphenyl dioxygenase from *Pseudomonas* results in a modification of the enzyme activity. *Biochem. Biophys. Res. Commun.*, 204:912–917.
360. Teramoto, M., Futamata, H., Harayama, S., and Watanabe, K., 1999, Characterization of a high-affinity phenol hydroxylase from *Comamonas testosteroni* R5 by gene cloning, and expression in *Pseudomonas aeruginosa* PAO1c. *Mol. Gen. Genet.*, 262:552–558.
361. Thiele, J., Müller, R., and Lingens, F., 1988, Enzymatic dehalogenation of 4-chlorobenzoate by 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. CBS3 in organic solvents. *Appl. Microbiol. Biotechnol.*, 27: 577–580.
362. Tiedje, J.M., Duxbury, J.M., Alexander, M., and Dawson, J.E., 1969, 2,4-D Metabolism: Pathway of degradation of chlorocatechols by *Arthrobacter* sp. *J. Agric. Food Chem.*, 17:1021–1026.
363. Tirola, M.A., Mannisto, M.K., Puhakka, J.A., and Kulomaa, M.S., 2002, Isolation and characterization of *Novosphingobium* sp. strain MT1, a dominant polychlorophenol-degrading strain in a groundwater bioremediation system. *Appl. Environ. Microbiol.*, 68:173–180.
364. Tirola, M.A., Wang, H., Paulin, L., and Kulomaa, M.S., 2002, Evidence for natural horizontal transfer of the *pcpB* gene in the evolution of polychlorophenol-degrading sphingomonads. *Appl. Environ. Microbiol.*, 68:4495–4501.
365. Top, E.M., Holben, W., and Forney, L.J., 1995, Characterization of diverse 2,4-dichlorophenoxyacetic acid-degradative plasmids isolated from soil by complementation. *Appl. Environ. Microbiol.*, 61:1691–1698.
366. Top, E.M., Springael, D., and Boon, N., 2002, Catabolic mobile genetic elements and their potential use in bioaugmentation of polluted soils and waters. *FEMS Microbiol. Ecol.*, 42:199–208.
367. Topp, E. and Akhtar, M.H., 1991, Identification and characterization of a *Pseudomonas* strain capable of metabolizing phenoxybenzoates. *Appl. Environ. Microbiol.*, 57:1294–1300.
368. Tsoi, T.V., Plotnikova, E.G., Cole, J.R., Guerin, W.F., Bagdasarian, M., and Tiedje, J.M., 1999, Cloning, expression, and nucleotide sequence of the *Pseudomonas aeruginosa* 142 *ohb* genes coding for oxygenolytic *ortho* dehalogenation of halobenzoates. *Appl. Environ. Microbiol.*, 65:2151–2162.
369. Vaillancourt, F.H., Haro, M.A., Drouin, N.M., Karim, Z., Maaroufi, H., and Eltis, L.D., 2003, Characterization of extradiol dioxygenases from a polychlorinated biphenyl-degrading strain that possess higher specificities for chlorinated metabolites. *J. Bacteriol.*, 185:1253–1260.
370. Vaillancourt, F.H., Labbe, G., Drouin, N.M., Fortin, P.D., and Eltis, L.D., 2002, The mechanism-based inactivation of 2,3-dihydroxybiphenyl 1,2-dioxygenase by catecholic substrates. *J. Biol. Chem.*, 277:2019–2027.
371. Vallaey, T., Fulthorpe, R.R., Wright, A.M., and Soulas, G., 1996, The metabolic pathway of 2,4-dichlorophenoxyacetic acid degradation involves different families of *tfdA* and *tfdB* genes according to PCR-RFLP analysis. *FEMS Microbiol. Ecol.*, 20:163–172.
372. van den Tweel, W.J.J., Kok, J.B., and de Bont, J.A.M., 1987, Reductive dechlorination of 2,4-dichlorobenzoate to 4-chlorobenzoate and hydrolytic dehalogenation of 4-chloro-, 4-bromo-, and 4-iodobenzoate by *Alcaligenes denitrificans* NTB1. *Appl. Environ. Microbiol.*, 53:810–815.
373. van der Meer, J.R., van Neerven, A.R.W., de Vries, E.J., de Vos, W.M., and Zehnder, A.J.B., 1991, Cloning and characterization of plasmid-encoded genes for the degradation of 1,2-dichloro-, 1,4-dichloro-, and 1,2,4-trichlorobenzene of *Pseudomonas* sp. strain P51. *J. Bacteriol.*, 173: 6–15.
374. van der Meer, J.R., Ravatn, R., and Senthilo, V., 2001, The *clc* element of *Pseudomonas* sp. strain B13 and other mobile degradative elements employing phage-like integrases. *Arch. Microbiol.*, 175:79–85.

375. van der Meer, J.R., Roelofsen, W., Schra, G., and Zehnder, A.J.B., 1987, Degradation of low concentrations of dichlorobenzenes and 1,2,4-trichlorobenzene by *Pseudomonas* sp. strain P51 in nonsterile soil columns. *FEMS Microbiol. Ecol.*, 45:333–341.
376. Vargas, C., Song, B., Camps, M., and Häggblom, M.M., 2000, Anaerobic degradation of fluorinated aromatic compounds. *Appl. Microbiol. Biotechnol.*, 53:342–347.
377. Vollmer, M.D., Hoier, H., Hecht, H.J., Schell, U., Groning, J., Goldman, A., and Schlömann, M., 1998, Substrate specificity of and product formation by muconate cycloisomerases: An analysis of wild-type enzymes and engineered variants. *Appl. Environ. Microbiol.*, 64:3290–3299.
378. Vollmer, M.D., Schell, U., Seibert, V., Lakner, S., and Schlömann, M., 1999, Substrate specificities of the chloromuconate cycloisomerases from *Pseudomonas* sp. B13, *Ralstonia eutropha* JMP134 and *Pseudomonas* sp. P51. *Appl. Microbiol. Biotechnol.*, 51:598–605.
379. Vollmer, M.D. and Schlömann, M., 1995, Conversion of 2-chloro-*cis,cis*-muconate and its metabolites 2-chloro- and 5-chloromuconolactone by chloromuconate cycloisomerase of pJP4 and pAC27. *J. Bacteriol.*, 177:2938–2941.
380. Vollmer, M.D., Stadler-Fritzsche, K., and Schlömann, M., 1993, Conversion of 2-chloromaleylacetate in *Alcaligenes eutrophus* JMP134. *Arch. Microbiol.*, 159:182–188.
381. Vollmer, M.D., Fischer, P., Knackmuss, H.-J., and Schlömann, M., 1994, Inability of muconate cycloisomerases to cause dehalogenation during conversion of 2-chloro-*cis,cis*-muconate. *J. Bacteriol.*, 176:4366–4375.
382. Weisshaar, M.P., Franklin, F.C., and Reineke, W., 1987, Molecular cloning and expression of the 3-chlorobenzoate-degrading genes from *Pseudomonas* sp. strain B13. *J. Bacteriol.*, 169:394–402.
383. Wen, A., Fegan, M., Hayward, C., Chakraborty, S., and Sly, L., 1999, Phylogenetic relationships among members of the Comamonadaceae, and description of *Delftia acidovorans* (den Dooren de Jong 1926 and Tamaoka *et al.*, 1987) gen. nov., comb. nov. *Int. J. Syst. Bacteriol.*, 49:567–576.
384. Werlen, C., Kohler, H.P.E., and van der Meer, J.R., 1996, The broad substrate chlorobenzene dioxygenase and *cis*-chlorobenzene dihydrodiol dehydrogenase of *Pseudomonas* sp. strain P51 are linked evolutionarily to the enzymes for benzene and toluene degradation. *J. Biol. Chem.*, 271:4009–4016.
385. Whited, G.M. and Gibson, D.T., 1991, Toluene-4-monooxygenase, a three-component enzyme system that catalyzes the oxidation of toluene to *p*-cresol in *Pseudomonas mendocina* KR1. *J. Bacteriol.*, 173:3010–3016.
386. Wieser, M., Wagner, B., Eberspächer, J., and Lingens, F., 1997, Purification and characterization of 2,4,6-trichlorophenol-4-monooxygenase, a dehalogenating enzyme from *Azotobacter* sp. strain GP1. *J. Bacteriol.*, 179:202–208.
387. Wilkes, H., Wittich, R.M., Timmis, K.N., Fortnagel, P., and Francke, W., 1996, Degradation of chlorinated dibenzofurans and dibenzo-*p*-dioxins by *Sphingomonas* sp. strain RW1. *Appl. Environ. Microbiol.*, 62:367–371.
388. Willems, A., Goor, M., Thielemans, S., Gillis, M., Kersters, K., and de Ley, J., 1992, Transfer of several phytopathogenic *Pseudomonas* species to *Acidovorax* as *Acidovorax avenae* subsp. *avenae* subsp. nov., comb. nov., *Acidovorax avenae* subsp. *citrulli*, *Acidovorax avenae* subsp. *cattleiae*, and *Acidovorax konjaci*. *Int. J. Syst. Microbiol.*, 42:107–119.
389. Williams, P.A., Jones, R.M., and Shaw, L.E., 2002, A third transposable element, ISPPu12, from the toluene-xylene catabolic plasmid pWW0 of *Pseudomonas putida* mt-2. *J. Bacteriol.*, 184:6572–6580.
390. Wittich, R.-M., Schmidt, S., and Fortnagel, P., 1990, Bacterial degradation of 3- and 4-carboxybiphenyl ether by *Pseudomonas* sp. NSS2. *FEMS Microbiol. Lett.*, 67:157–160.
391. Wittich, R.-M., Wilkes, H., Sinnwell, V., Francke, W., and Fortnagel, P., 1992, Metabolism of dibenzo-*p*-dioxin by *Sphingomonas* sp. strain RW1. *Appl. Environ. Microbiol.*, 58:1005–1010.

392. Wittich, R.M., Strömpl, C., Moore, E.R.B., Blasco, R., and Timmis, K.N., 1999, Interaction of *Sphingomonas* and *Pseudomonas* strains in the degradation of chlorinated dibenzofurans. *J. Ind. Microbiol. Biotechnol.*, 23:353–358.
393. Wolgel, S.A., Dege, J.E., Perkins-Olson, P.E., Juarez-Garcia, C.H., Crawford, R.L., Münck, E., and Lipscomb, J.D., 1993, Purification and characterization of protocatechuate 2,3-dioxygenase from *Bacillus macerans*: A new extradiol catecholic dioxygenase. *J. Bacteriol.*, 175:4414–4426.
394. Worsey, M. and Williams, P.A., 1975, Metabolism of toluene and xylenes by *Pseudomonas putida* (arvilla) mt-2: Evidence for a new function of the TOL plasmid. *J. Bacteriol.*, 124:7–13.
395. Xiang, H., Luo, L.S., Taylor, K.L., and Dunaway-Mariano, D., 1999, Interchange of catalytic activity within the 2-enoyl-coenzyme A hydratase isomerase superfamily based on a common active site template. *Biochemistry*, 38:7638–7652.
396. Xu, L., Resing, K., Lawson, S.L., Babbitt, P.C., and Copley, S.D., 1999, Evidence that *pcpA* encodes 2,6-dichlorohydroquinone dioxygenase, the ring cleavage enzyme required for pentachlorophenol degradation in *Sphingomonas chlorophenolica* strain ATCC 39723. *Biochemistry*, 38:7659–7669.
397. Xun, L. and Orser, C.S., 1991, Purification and properties of pentachlorophenol hydroxylase, a flavoprotein from *Flavobacterium* sp. strain ATCC 39723. *J. Bacteriol.*, 173:4447–4453.
398. Xun, L., Topp, E., and Orser, C.S., 1992, Purification and characterization of a tetrachloro-*p*-hydroquinone reductive dehalogenase from a *Flavobacterium* sp. *J. Bacteriol.*, 174:8003–8007.
399. Xun, L.Y., 1996, Purification and characterization of chlorophenol 4-monooxygenase from *Burkholderia cepacia* AC1100. *J. Bacteriol.*, 178:2645–2649.
400. Xun, L.Y., Bohuslavsek, J., and Cai, M.A., 1999, Characterization of 2,6-dichloro-*p*-hydroquinone 1,2-dioxygenase (PcpA) of *Sphingomonas chlorophenolica* ATCC 39723. *Biochem. Biophys. Res. Commun.*, 266:322–325.
401. Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T., and Arakawa, M., 1992, Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. *Microbiol. Immunol.*, 36:1251–1275.
402. Yabuuchi, E., Kosako, Y., Yano, I., Hotta, H., and Nishiuchi, Y., 1995, Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. nov.; proposal of *Ralstonia pickettii* (Ralston, Palleroni, and Douderoff, 1973) comb. nov., *Ralstonia solanacearum* (Smith, 1896) comb. nov. and *Ralstonia eutropha* (Davis, 1969) comb. nov. *Microbiol. Immunol.*, 39:897–904.
403. Yamamoto, S., Katagiri, M., Maeno, H., and Hayaishi, O., 1965, Salicylate hydroxylase, a monooxygenase requiring flavin adenine dinucleotide. I. Purification and general properties. *J. Biol. Chem.*, 240:3408–3413.
404. Yang, G., Liang, P.-H., and Dunaway-Mariano, D., 1994, Evidence of nucleophilic catalysis in the aromatic substitution reaction catalyzed by (4-chlorobenzoyl) coenzyme A dehalogenase. *Biochemistry*, 33:8527–8531.
405. Yeh, W.K., Gibson, D.T., and Liu, T.-N., 1977, Toluene dioxygenase: A multicomponent enzyme system. *Biochem. Biophys. Res. Commun.*, 78:401–411.
406. Yen, K.-M. and Karl, M.R., 1992, Identification of a new gene, *tmoF*, in the *Pseudomonas mendocina* KR1 gene cluster encoding toluene-4-monooxygenase. *J. Bacteriol.*, 174:7253–7261.
407. Yen, K.-M., Karl, M.R., Blatt, L.M., Simon, M.J., Winter, R.B., Fausset, P.R., Lu, H.S., Harcourt, A.A., and Chen, K.K., 1991, Cloning and characterization of a *Pseudomonas mendocina* KR1 gene cluster encoding toluene-4-monooxygenase. *J. Bacteriol.*, 173:5315–5327.
408. Yen, K.M. and Gunsalus, I.C., 1982, Plasmid gene organization: Naphthalene/salicylate oxidation. *Proc. Natl. Acad. Sci. USA*, 79:874–878.

409. You, I.-S., Ghosal, D., and Gunsalus, I.C., 1991, Nucleotide sequence analysis of the *Pseudomonas putida* PpG7 salicylate hydroxylase gene (*nahG*) and its 3'-flanking region. *Biochemistry*, 30:1635–1641.
410. Zaborina, O., Daubaras, D.L., Zago, A., Xun, L.Y., Saido, K., Klem, T., Nikolic, D., and Chakrabarty, A.M., 1998, Novel pathway for conversion of chlorohydroxyquinol to maleylacetate in *Burkholderia cepacia* AC1100. *J. Bacteriol.*, 180:4667–4675.
411. Zaitsev, G.M. and Karasevich, Y.N., 1984, Utilization of 2-chlorobenzoic acid by *Pseudomonas cepacia*. *Mikrobiologiya*, 53:75–80.
412. Zhou, J. and Tiedje, J.M., 1995, Gene transfer from a bacterium injected into an aquifer to an indigenous bacterium. *Mol. Ecol.*, 4:613–618.
413. Zielinski, M., Backhaus, S., and Hofer, B., 2002, The principal determinants for the structure of the substrate-binding pocket are located within a central core of a biphenyl dioxygenase alpha subunit. *Microbiology*, 148:2439–2448.
414. Zylstra, G.J., McCombie, W.R., Gibson, D.T., and Finette, B.A., 1988, Toluene degradation by *Pseudomonas putida* F1: Genetic organization of the *tod* operon. *Appl. Environ. Microbiol.*, 54:1498–1503.

CATABOLISM OF NITROAROMATIC COMPOUNDS

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1. INTRODUCTION

Nitroaromatic compounds, though rare in nature, are versatile and favored tools of the synthetic chemist and became widely distributed in the biosphere after the advent of the industrial revolution. Compounds such as nitrobenzene (NB) consistently rank among the most commonly used industrial chemicals in the world, because it is the gateway to the production of aniline and thus to dyes, resins, inks, and rubber. Dinitrotoluene (DNT) is similarly the precursor to toluenediisocyanate which in turn is the major monomer used to manufacture polyurethane foams, elastomers, and coatings. 2,4,6-Trinitrotoluene (TNT) became the most widely used military explosive in the world shortly after the development of practical methods to manufacture substantial quantities of the explosive. Other nitroaromatic compounds have gained widespread use as pesticides and herbicides.

The sudden addition of nitroaromatic compounds to the biosphere provided the indigenous microbial populations with new sources of carbon, nitrogen, and electron acceptors. But the bacteria had to adapt and develop new catabolic pathways before the novel substrates could be exploited for growth. Pseudomonads are opportunistic bacteria characterized by metabolic flexibility, the ability to acquire and incorporate foreign DNA, and a large genome to provide the necessary raw materials for recruitment and evolution of new pathways. During the past half-century, a number of pseudomonads with the ability to grow on nitroaromatic compounds have been isolated from soils and ground

water contaminated with nitroaromatic compounds (Table 1). The challenge for bacteria has been to overcome the electron-withdrawing capacity of the nitro group and break the aromatic ring. The more highly nitro-substituted the benzene ring, the more recalcitrant the nitroaromatic molecule is to attack by the oxygenase enzymes that are typically involved in aerobic degradation of aromatic compounds^{119a}.

As our detailed knowledge of bacterial catabolism of nitroaromatic compounds has increased, it has become apparent that there are several recurring themes among the mechanisms that account for most of the initial steps in the aerobic catabolic pathways (Figure 1). The themes can be summarized as: (a) partial reduction of the nitro group followed by rearrangement, (b) reduction of the aromatic ring, and (c) ring hydroxylation with the release of nitrite. To date only aerobic or microaerophilic bacteria are known to use nitroaromatic compounds as growth substrates.

The chemistry of the nitro group allows facile reduction either by nitroreductases or abiotic reduction by a variety of electron donors including metals. Reductive reactions can take place either aerobically or anaerobically. Nonetheless, because nonspecific reduction provides little selective advantage, bacteria that mineralize or grow on nitroaromatic compounds via reductive pathways do so aerobically. The reductive pathways that lead to mineralization stop short of the amine at the hydroxylamino intermediate. Then either a mutase or a lyase transforms the hydroxylamino compound to an aminophenol or a dihydroxy compound. In contrast, bacteria that use nonspecific nitroreductases to reduce nitroaromatic compounds to aminoaromatic compounds accumulate the amines as dead end metabolites.

Reduction of the aromatic ring leading to the formation of a Meisenheimer complex is the initial reaction in the degradation of di- and trinitrophenols. Meisenheimer complexes have also been reported for TNT, but formation of the TNT-Meisenheimer complex does not then lead to mineralization.

Monooxygenases typically catalyze the replacement of the nitro group of simple nitrophenols by a hydroxyl group. Dioxygenases catalyze the initial attack on nonpolar mono- and dinitroaromatic compounds. Regardless of the initial mode of attack, the subsequent degradation pathways generally involve *meta*-ring cleavage catalyzed by dioxygenases. For mononitroaromatic compounds, the lower pathways are often the familiar pathways known from degradation pathways for the simpler aromatic compounds. For di- and trinitroaromatic compounds, more unusual pathways have evolved to deal with the additional nitro groups.

Pathways for degradation of novel compounds, including nitroaromatic compounds, arise through well-recognized processes of gene transfer, genetic mutation, and recombination and transposition. A number of reviews

Table 1. Nitroaromatic compounds that serve as growth substrates for bacteria.

Compound	Strain	Mechanism	References
Nitrobenzene	<i>Pseudomonas pseudoalcaligenes</i> JS45	Partial reduction (mutase)	[26], [27], [58]–[62], [64], [90], [110], [141]
	<i>Comamonas</i> sp. JS765	Dioxygenation	[62], [95], [111], [119]
1,3-Dinitrobenzene 2-Nitrobenzoate	<i>Pseudomonas putida</i> HS12	Partial reduction (mutase)	[120]–[122]
	<i>Pseudomonas</i> sp. JX165	Partial reduction (mutase)/amine	[160]
	<i>Rhodococcus</i> sp. QT-1	Dioxygenation	[30]
	<i>Pseudomonas fluorescens</i> KU-7	Partial reduction (mutase)	[104]
	<i>Pseudomonas pseudoalcaligenes</i>	ND*	[103]
	Various <i>Nocardia</i>	ND	[15], [16]
	<i>Arthrobacter protophormiae</i> RKJ100	ND	[19]
	<i>Burholderia</i> sp. JS51	Dioxygenation	[107]
	<i>Comamonas</i> sp. JS46	Dioxygenation	[43], [107]
	<i>Comamonas</i> sp. JS47	ND	[43]
3-Nitrobenzoate 4-Nitrobenzoate	<i>Comamonas acidovorans</i> NBA-10	Partial reduction (hydroxylaminolase)	[44], [45]
	<i>Burkholderia cepacia</i> PB4	Partial reduction (hydroxylaminolase)	[123]–[125]
	<i>Ralstonia paucula</i> SB4	Partial reduction (hydroxylaminolase)	[124]
	<i>Ralstonia pickettii</i> YH105	Partial reduction (hydroxylaminolase)	[165]
	<i>Pseudomonas fluorescens</i> 410PR	Partial reduction (hydroxylaminolase)	[102]
2-Nitrophenol	<i>Nocardia</i>	ND	[15], [16]
	<i>Pseudomonas putida</i> B2	Monooxygenation	[167]–[169]

Table 1. Continued

Compound	Strain	Mechanism	References
3-Nitrophenol	<i>Pseudomonas putida</i> B2	Partial reduction (hydroxylaminolase)	[101], [167]
	<i>Ralstonia eutropha</i> JMP134	Partial reduction (mutase)	[137], [138]
	<i>Pseudomonas putida</i> 2NP8	Partial reduction (mutase)	[170], [171]
4-Nitrophenol	<i>Moraxella</i> sp.	Monoxygenation	[145], [147]
	<i>Pseudomonas putida</i> JS444	Monoxygenation	[109]
	<i>Arthrobacter</i> sp. JS443	Monoxygenation	[75]
	<i>Burkholderia cepacia</i> RKJ200	Monoxygenation	[9], [20], [126]
	<i>Bacillus sphaericus</i> JS905	Monoxygenation	[84], [85]
	<i>Ralstonia</i> sp. SJ98	Monoxygenation	[9], [134]
4-Nitrocatechol	<i>Sphingomonas</i> sp. UG30	Monoxygenation	[17], [97], [166]
2,4-Dinitrophenol	<i>Rhodococcus</i> sp. RB1	Ring reduction	[10]
	<i>Rhodococcus erythropolis</i> HL-1	Ring reduction	[93], [94]
2,6-Dinitrophenol	<i>Ralstonia eutropha</i> JMP134	Dioxygenation	[33]
2,4,6-Trinitrophenol	<i>Nocardiodex</i> sp. CB 22-2	Ring reduction	[8]
	<i>Nocardiodex simplex</i> FJ2-1A	Ring reduction	[32]
	<i>Rhodococcus (opacus) erythropolis</i> HL PM-1	Ring reduction	[65]
2-Nitrotoluene	<i>Acidovorax</i> sp. JS42	Dioxygenation	[5], [52], [115], [116]
3-Nitrotoluene	<i>Pseudomonas putida</i> OU83	ND	[4]
	<i>Acidovorax</i> sp. JS42	Dioxygenation	
4-Nitrotoluene	<i>Pseudomonas</i> sp. 4NT	Dioxygenation	[50]
	<i>Mycobacterium</i> sp. HL 4-NT-1	Partial reduction (hydroxylaminolase)	
	<i>Pseudomonas putida</i> TW3	Partial reduction (mutase)	[64], [149]
		Partial reduction (hydroxylaminolase)	[72], [73], [76], [77], [131]

2,4-Dinitrotoluene	<i>Pseudomonas fluorescens</i> 410PR(pWW0Dpm) [†]	Partial reduction (hydroxylaminolase)	[102]
	<i>Burkholderia</i> sp. DNT	Dioxygenation	[49], [148]
	<i>Burkholderia cepacia</i> JS872	Dioxygenation	[112]
	<i>Burkholderia cepacia</i> R34	Dioxygenation	[80], [81]
2,6-Dinitrotoluene	<i>Hydrogenophaga palleronii</i> JS863	Dioxygenation	[108], [112]
2,4,6-Trinitrotoluene	<i>Pseudomonas</i> sp. JLR11	Mixed [‡] , anaerobic	[35], [36]
Methyl parathion	<i>Pseudomonas putida</i>	Monoxygenation	[130]
	<i>Pseudomonas</i> sp. A3	Monoxygenation	[129]
4-Nitroanisole	<i>Rhodococcus</i> sp.	Monoxygenation	[136]
2-Chloro-5-nitrophenol	<i>Ralstonia eutropha</i> JMP134	Partial reduction (mutase)	[139]
4-Chloro-2-nitrophenol	<i>Pseudomonas</i> sp. N31	Monoxygenation	[14]
4-Chloronitrobenzene	<i>Comamonas</i> strain LW1	Partial reduction (mutase)	[86]
4,6-Dinitro- <i>o</i> -cresol	Various bacteria	Reduction	[53], [155]
2-sec-butyl- 4,6-dinitrophenol	Various bacteria <i>Clostridium bifermentans</i> KMR-1	Hydroxylation ND, anaerobic	[46], [78] [54]

*ND = not determined or not reported.

[†]Hybrid strain.

[‡]Mixed, compound serves only as a nitrogen source, aromatic ring not utilized.

Strains in bold are pseudomonads or former pseudomonads.

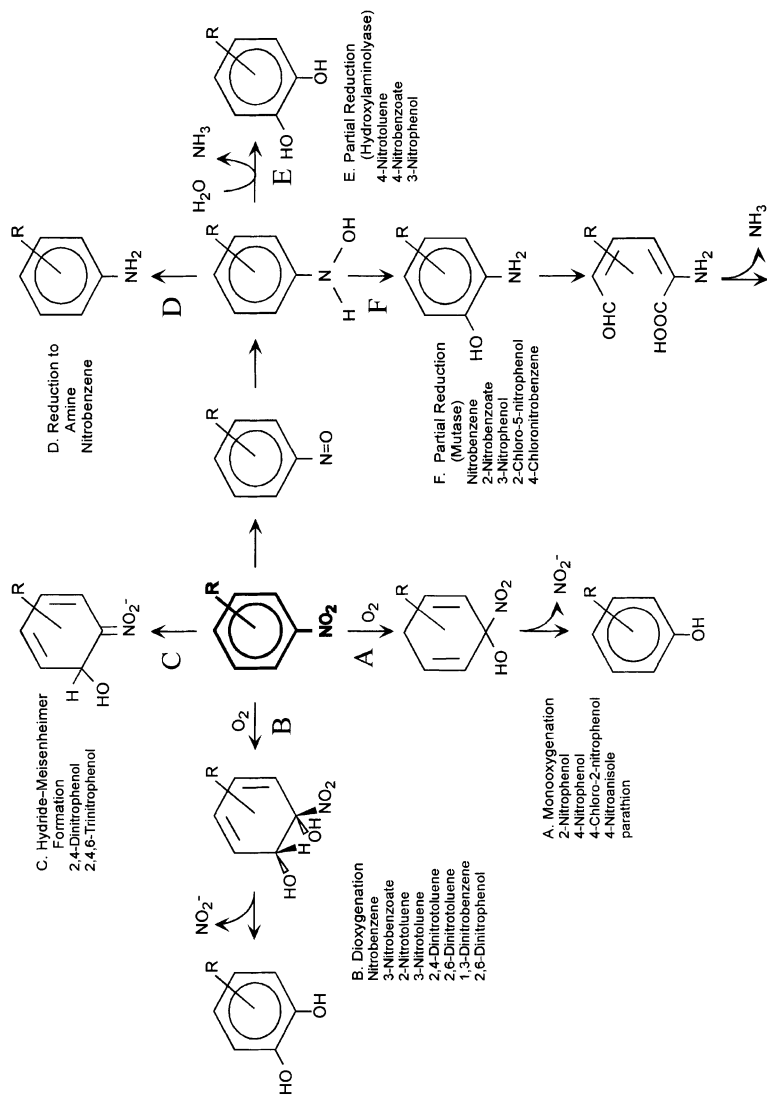


Figure 1. Mechanisms for the biodegradation of nitroaromatic compounds. A. Monoxygenation, B. dioxygenation, C. hydride-Meisenheimer formation, D. reduction to the amine, E. partial reduction followed by intramolecular rearrangement by a lyase, and F. partial reduction followed by intramolecular rearrangement by a mutase.

deal with both general and specific aspects of the evolution of catabolic pathways^{7, 21, 28, 55, 57, 74, 89, 98, 157, 162, 164}. Specific instances where we have some understanding of the evolution of pathways for the degradation of nitroaromatic compounds will be discussed throughout this chapter.

2. MECHANISMS AND PATHWAYS FOR CATABOLISM OF NITROAROMATIC COMPOUNDS

The mechanisms for the initial attack on nitroaromatic compounds briefly introduced above will be described in more detail and illustrated with examples of pathways elucidated in pseudomonads. Comprehensive reviews of biodegradation and biotransformation of nitroaromatic compounds by all microorganisms can be found in recent books^{142, 146} and elsewhere^{11, 34, 143, 156}.

2.1. Reduction of Nitro Groups

Nitroreductase enzymes catalyze the reduction of nitro groups in bacterial systems. Complete reduction proceeds stepwise via either 1-electron or 2-electron reduction through nitroso and hydroxylamino intermediates to an amine^{22, 88}. With few exceptions, reduction of the nitro group stops at the hydroxylamino level in the productive aerobic pathways. At that point the pathways diverge and the next reactions are rearrangements catalyzed by a mutase, or by a lyase. Reduction only to the hydroxylamine has been explained in terms of the thermodynamic properties of a nitroreductase from *Enterobacter cloacae*⁸⁸. Reduction to the level of the amine is predominantly catalyzed by nonspecific nitroreductases under cometabolic and, often, anaerobic conditions. Such nonspecific nitroreductases whose physiological functions are unknown, but are probably quinone reductases, are widespread among the pseudomonads¹³⁵. Although reduction of the nitro group to the amine is frequently cited as a mechanism for making nitroaromatic compounds less toxic, the reactive intermediates are toxic, mutagenic, and carcinogenic^{18, 99}. The free radicals that have been implicated in the toxic effects can be produced either directly during 1-electron reduction, or indirectly by mild reduction of nitroso intermediates⁹⁹. The reductive degradation pathways used for growth on nitroaromatic compounds include enzymes that rapidly remove the products of the nitroreductase-catalyzed reactions so that partially reduced reactive intermediates never accumulate.

Facile reduction of the nitro group is attributed to its high electronegativity¹²⁸. In general, more nitro groups on the aromatic ring make

compounds more readily reduced. Conversely, more amino groups make compounds more difficult to reduce. Therefore, the reduction of one or two nitro groups on a polynitroaromatic compound might take place under aerobic conditions, but reduction of three nitro groups only proceeds under strictly anaerobic conditions¹²⁷.

2.1.1. Nitrobenzene Degradation via a Mutase Catalyzed Bamberger Rearrangement

Nitrobenzene is the simplest of the nitroaromatic compounds, yet the degradation pathway has proved to be one of the most interesting. The prototypical pathway for partially reductive degradation of NB was discovered in *Pseudomonas pseudoalcaligenes* JS45¹¹⁰. The initial attack on the nitro group is catalyzed by a specific NB nitroreductase. The molecule is reduced to nitrosobenzene, then in turn to hydroxylaminobenzene. The key enzyme in the pathway is a mutase that converts hydroxylaminobenzene to 2-aminophenol via a Bamberger-like intramolecular rearrangement. A *meta*-ring cleavage yields 2-aminomuconic semialdehyde which is oxidized to 2-aminomuconic acid⁶¹. Deamination yields 4-oxalocrotonic acid which is degraded to pyruvate and acetaldehyde (Figure 2). The same NB degradation pathway operates in *Pseudomonas putida* HS12¹²⁰ and *Pseudomonas* sp. JX165¹⁶⁰. A pathway in *Pseudomonas* sp. AP-3 for 2-aminophenol degradation has been well characterized^{6, 152–154}. Analogous initial reactions are involved in the degradation of more substituted nitroaromatic compounds including: 2-nitrobenzoate by *Pseudomonas fluorescens* KU-7¹⁰⁴, 3-nitrophenol by *P. putida* 2NP8^{170, 171} and *Ralstonia eutropha* JMP134¹³⁷, 1-chloro-4-NB by comamonad strain LW1⁸⁶, and 4-nitrotoluene by *Mycobacterium* sp. HL-4-NT-1¹⁴⁹.

The first three enzymes of the pathway were unusual and were, therefore, investigated in detail. NB nitroreductase from JS45 was the first of the enzymes to be purified and characterized¹⁴¹ and remains the only NB nitroreductase purified to date. The flavoprotein NB nitroreductase reduces the nitro group of a variety of nitroaromatic compounds to the corresponding hydroxylamino compounds^{37, 83}. In addition to the specific NB nitroreductase, there is also evidence for nonspecific nitroreductases in *P. pseudoalcaligenes* JS45 that can reduce nitro groups to the amine, but the activities are much lower³⁷. *nbzA*, the gene that encodes NB nitroreductase, has recently been cloned, but the sequence has not as yet been published (Zylstra, personal communication). *nbzA* from *P. putida* HS12 (Table 2) is carried on a plasmid that also carries the 2-aminophenol degradation operon, but not the hydroxylaminobenzene mutase^{120, 121}. The nitroreductase from JS45 is inducible¹¹⁰, but the activity appears to be constitutive in HS12¹²⁰. The explanation of the difference in the regulation in the two strains awaits further investigation. NbzA is distantly related to other flavoprotein reductases⁸² but the immediate progenitor is unknown.

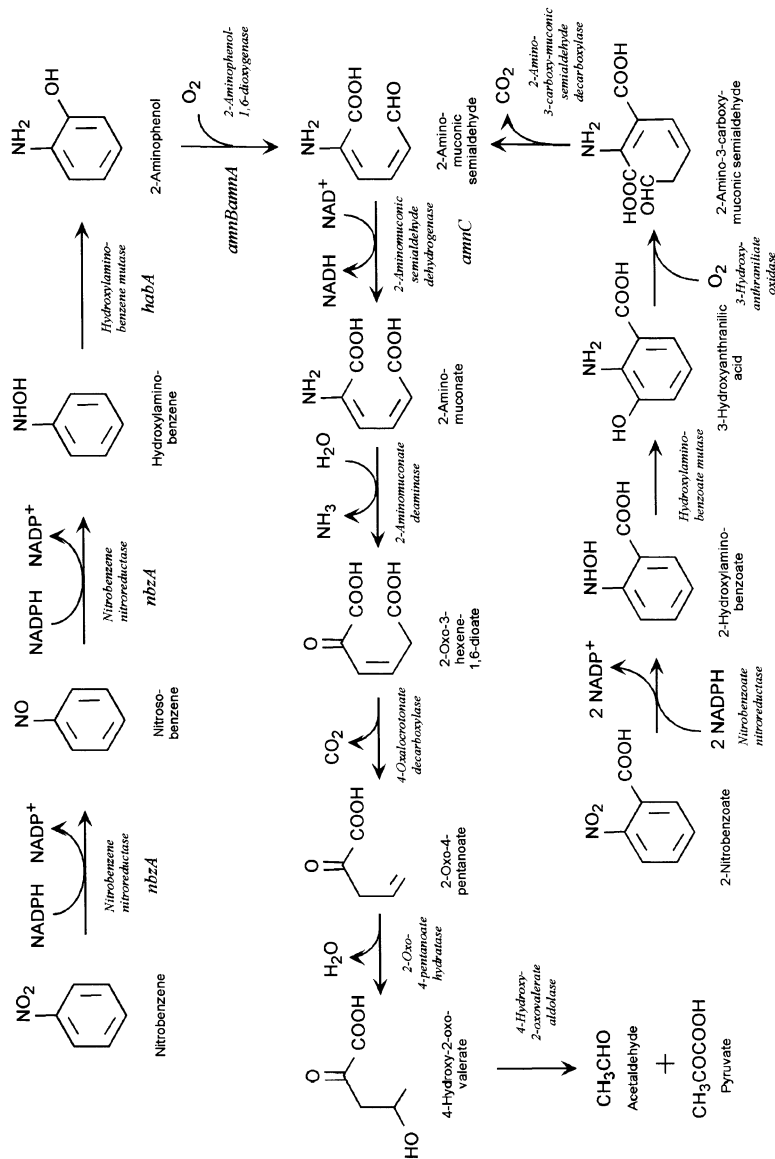


Figure 2. Partial reductive pathways for NB and 2-nitrobenzoate proceed via mutase catalyzed rearrangements. Genes indicated are from *P. pseudocaligenes* JS45.

Table 2. Genes that have been cloned and sequenced from mutase-containing pathways.

Enzyme	Strain JS45 (nitrobenzene)	Strain AP-3 (2-aminophenol)	Strain HS12 (nitrobenzene)	Strain KU-7 (2-nitrobenzoate)
Nitrobenzene nitroreductase	<i>nbzA</i>		<i>nbzA</i>	<i>nbaA</i>
Hydroxylaminobenzene mutase	<i>habA</i>		<i>nbzB</i>	<i>nbaB</i>
2-Aminophenol-1,6-dioxygenase	<i>amnBA</i>	<i>amnBA</i>	<i>nbzCab</i>	<i>nbaC</i>
2-Nitrobenzoate decarboxylase				<i>nbaD</i>
2-Aminomuconic semialdehyde dehydrogenase	<i>amnC</i>	<i>amnC</i>	<i>nbzD</i>	<i>nbaE</i>
2-Aminomuconate deaminase		<i>amnD</i>	<i>nbzE</i>	<i>nbaF</i>
4-Oxalocrotonate decarboxylase		<i>amnE</i>	<i>nbzF</i>	<i>nbaG</i>
2-Oxo-4-pentanoate hydratase		<i>amnF</i>	<i>nbzG</i>	<i>nbaH</i>
4-Hydroxy-2-oxovalerate aldolase		<i>amnG</i>	<i>nbzH</i>	<i>nbaI</i>
Acetaldehyde dehydrogenase		<i>amnH</i>	<i>nbzI</i>	<i>nbaJ</i>
Putative ferredoxin/YjgF-like	ORF1		<i>nbzJ</i>	<i>nbaX</i>
Regulatory			<i>nbzR</i>	
Regulatory				<i>nbaR</i>

Genes on the same lines in bold share significant sequence identity.

The mutase that converts hydroxylaminobenzene to 2-aminophenol is the key enzyme in the pathway. It transforms a potentially reactive intermediate into a compound that can be degraded via a well organized and conserved pathway⁸². H_2^{18}O studies have shown that the intramolecular rearrangement shifts the hydroxyl group from the hydroxylamino moiety to an *ortho* position on the aromatic ring^{59, 106} (Figure 3). Detailed investigation of the mechanism is hampered by the lack of purified enzymes. The mutase from JS45 is active against a variety of *ortho*-, *meta*-, and *para*-substituted hydroxylaminobenzenes^{83, 106}. The mutases described to date^{86, 104, 122, 138, 149} generally catalyze the production of *ortho*-aminophenols from their physiological substrates and various substituted hydroxylaminobenzenes. The mutase from the 3-nitrophenol degrading bacterium, *R. eutropha* JMP134, is an exception¹³⁸. The JMP134 mutase converts its physiological substrate to an *ortho*-aminophenol, but converts hydroxylaminobenzene to both 4- and 2-aminophenol, in a 1.7:1 ratio¹³⁷. A mechanism to explain the different outcomes has been proposed¹⁰⁶. A mutase from another 3-nitrophenol degrading bacterium, *P. putida* 2NP8, also catalyzes the conversion of the hydroxylaminobenzene to both

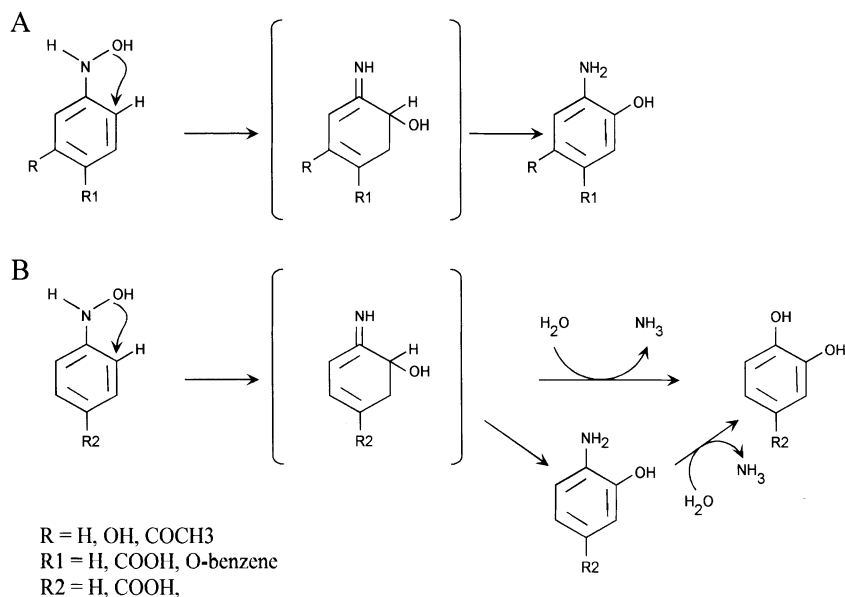


Figure 3. Intramolecular rearrangements catalyzed by A. mutases and B. lyases (see next section). Substrates for hydroxylaminomutases are: hydroxylaminobenzene, 3-hydroxylaminophenol, 4-hydroxylaminobenzoate, 4-hydroxylaminobiphenyl ether, and hydroxylaminoacetophenones. Hydroxylaminolyase catalyzes rearrangements of hydroxylaminobenzene and 4-hydroxylaminobenzoate.

4- and 2-aminophenol^{170, 171}, but there is no information on the products of conversion of the physiological substrate, 3-hydroxylaminophenol. It is tempting to speculate that the two mutases are related, but the mutase from 2NP8 requires thorough characterization before any detailed comparison can be made. The authors also present a novel pathway for release of ammonia¹⁷⁰, but the scheme is based entirely on intermediates derived from other nitroaromatic compounds and not from any detected during 3-nitrophenol degradation.

The *habA* and *habB* genes from *P. pseudoalcaligenes* JS45 have been cloned and expressed in *Escherichia coli*²⁷. Both the genes encode proteins that catalyze the same intramolecular rearrangement¹⁰⁶, however only the HabA protein is expressed in the wild-type *P. pseudoalcaligenes* JS45²⁷. The HabA and HabB proteins differ significantly in size, thermostability, and predicted isoelectric point and are 44% identical. The only other mutase to be thoroughly characterized to date is the mutase from *R. eutropha* JMP134¹³⁸. It is a soluble protein several times larger than the HabA and HabB proteins from JS45, and with a much lower isoelectric point. The physical differences seem to underlie the differences in the reactivities of the mutases, but until more mutases are characterized, conclusions must be drawn with caution.

2-Aminophenol 1,6-dioxygenase catalyzes *meta*-ring cleavage of 2-aminophenol to form 2-aminomuconic semialdehyde. The subsequent steps in the pathway are essentially identical to those for 2-aminophenol degradation in *Pseudomonas* sp. AP-3^{6, 152–154} and converge with the pathway for 2-nitrobenzoate degradation in *P. fluorescens* KU-7¹⁰⁴. There is substantial similarity among the genes for the NB, 2-aminophenol, and 2-nitrobenzoate pathways (Table 2). The biochemistry is analogous to the catechol *meta*-cleavage pathway but the homology of the genes is more difficult to establish⁶². Selected enzymes have been purified only from JS45 and AP-3, and although extensive characterization is lacking for many of them, the overall similarities of the enzymes from the two pathways are striking. For the most part, the size of the native proteins and subunits from the two bacteria are very similar. The greatest difference appears to be between the 2-aminomuconate deaminases, the enzyme from JS45 is a homohexamer whereas that from AP-3 is a homotetramer, although the subunit sizes are very similar at 16.6 and 16 kDa. No conclusions can be drawn as to the meaning of this difference because of the lack of functional characterization of the enzyme from AP-3.

The 4-oxalocrotonate decarboxylase and 2-oxo-4-pentanoate hydratase have only been purified from AP-3¹⁵². The enzymes co-eluted through all purification steps and formed a complex at a constant ratio of 1:1. The mass of the native complex was 300 kDa. A similar complex of the two enzymes was purified from bacteria containing the TOL plasmid pWW0⁵⁶. Separation of the enzymes was only achieved by purification from clones.

Evolution of the NB degradation pathway has recently been reviewed⁸². The pathway appears to have been assembled from three separate sources. The NB nitroreductase, the hydroxylaminobenzene mutase, and the 2-aminophenol pathway have been recruited and assembled into a pathway. The best understood portion of the pathway is the 2-aminophenol degradation operon. The genetic organization is identical for the three pathways for which the genes have been completely sequenced in the 2-aminophenol-degrading *Pseudomonas* sp. TW-3, the NB-degrading *P. putida* HS12, and in the 2-nitrobenzoate-degrading *P. fluorescens* KU-7. The existence of a highly evolved system for degradation of 2-aminophenol can perhaps be attributed to long-term microbial exposure to naturally occurring 2-aminophenol⁸² as well as more recent intensive selection pressure from industrial use. The genes that encode NB nitroreductases from *P. pseudoalcaligenes* JS45 and *P. putida* HS12 have been sequenced and share high identity with each other but are not closely related to other nitroarene nitroreductases. How they came to be recruited into their respective pathways is unknown. The hosts were isolated from widely separated geographic locales, and in JS45 the gene is chromosomally encoded, whereas in HS12 the gene is carried on a plasmid. The phylogenies of the hydroxylaminobenzene mutases from the two NB-degrading strains are equally problematic. They share close identity only with each other, the non-coding *habB* mutase gene from JS45 and two other unknown open reading frames identified from *Mycobacterium* and *Novosphingobium* genomes. In both JS45 and HS12, the hydroxylaminobenzene mutase gene is in a separate operon from the nitroreductase gene and the genes of the 2-aminophenol degradation pathway. In HS12, the mutase gene is on a separate plasmid from all other genes for NB degradation. Regulation of the pathways is not yet understood.

2.1.2. 4-Nitrotoluene and 4-Nitrobenzoate Degradation via Hydroxylaminolyase

Partial reduction to the hydroxylamino level followed by rearrangement and ring-cleavage was first reported for degradation of 4-nitrobenzoate by *Comamonas acidovorans* NBA-10^{44, 45}. The rearrangement step is catalyzed by a hydroxylaminolyase with the addition of a hydroxyl group from water and the loss of ammonia (Figure 4). The pathway has since been found in *Comamonas* sp. JS47⁴³, *Burkholderia cepacia* PB4¹²³⁻¹²⁵, *Ralstonia paucula* SB4¹²⁴, *Ralstonia pickettii* YH105¹⁶⁵, and *P. fluorescens* 410PR¹⁰². Pathways for 4-nitrotoluene degradation via 4-nitrobenzoate were described in *P. putida* TW3¹³¹, and in *Pseudomonas* sp. 4NT⁵⁰. The initial attack on 4-nitrotoluene involves oxidation of the methyl group in a series of reactions analogous to those of the TOL pathway to form 4-nitrobenzoate. Indeed the upper pathway enzymes of the TOL pathway will convert 3- and 4-nitrotoluene to the appropriate nitrobenzoic

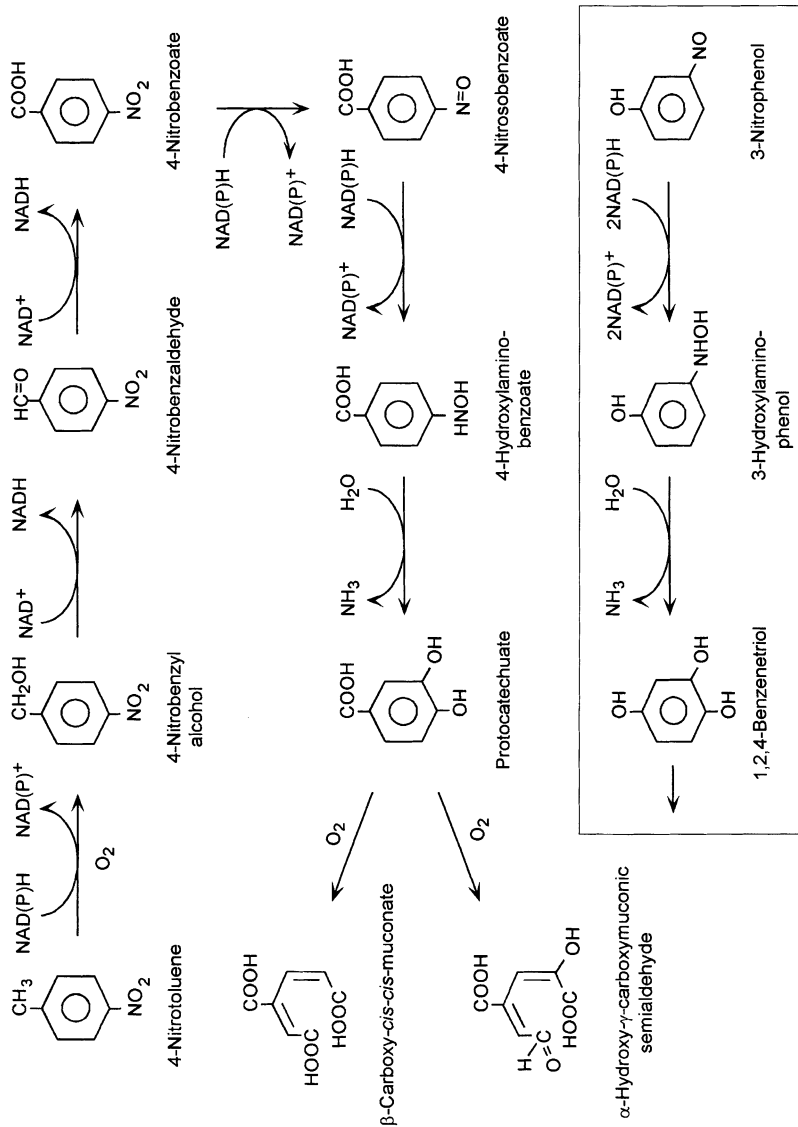


Figure 4. Degradation of 4-nitrobenzoate, 4-nitrotoluene, and 3-nitrophenol (inset) via a hydroxylaminolyase catalyzed rearrangement.

acids^{29, 102}, and a hybrid pathway to degrade 4-nitrotoluene via 4-nitrobenzoate has been constructed by moving the TOL plasmid into *P. fluorescens* 410PR¹⁰². The only pathway with a hydroxylaminolyase that does not involve 4-nitrobenzoate is a pathway proposed for the degradation of 3-nitrophenol in *P. putida* B2¹⁰¹ (Figure 4, inset).

Hydroxylaminolyases and hydroxylaminobenzene mutase are able to catalyze the formation of hydroxy aromatic compounds without the involvement of molecular oxygen¹⁰¹. Intermolecular rearrangement mechanisms were originally proposed to account for the observation^{100, 138}. An H₂¹⁸O study with HabB from JS45 cloned into *E. coli* unequivocally showed the involvement of an intramolecular rearrangement⁵⁹. The mechanism by which hydroxylaminolyases effect the rearrangement of hydroxylaminobenzenes is still not completely understood, but the major question of whether the rearrangement involved is intramolecular or intermolecular has recently been resolved with additional H₂¹⁸O studies¹⁰⁶.

Both enzymes appear to catalyze an initial intramolecular rearrangement in which the hydroxyl moiety from the hydroxylamino group migrates to the adjacent position on the aromatic ring to form an imine (Figure 3). Loss of H accompanied by rearomatization of the ring yields *o*-aminophenols in all the mutase-catalyzed reactions, and in the special case when hydroxylaminobenzene is the starting substrate for the hydroxylaminolyase-catalyzed reaction. When 4-hydroxylaminobenzoate, the physiological substrate for the lyase, is the starting substrate, the final product is protocatechuate¹⁰⁶. The mechanism for the conversion of the imine to protocatechuate is still open to investigation. Experimental evidence exists for two possible mechanisms: direct addition of water with the loss of ammonia to produce protocatechuate¹⁰⁶, or rearomatization to 4-amino-3-hydroxybenzoate followed by addition of water and loss of ammonia⁷².

Genes for degradation of 4-nitrobenzoate have been cloned from *R. pickettii* YH105^{165, 173}, *Pseudomonas* sp. YH102¹⁷³, and from *P. putida* TW3 (73). In addition, the genes that encode the upper pathway for the transformation of 4-nitrotoluene to 4-nitrobenzoate have been cloned from strain TW3^{76, 77}. 4-Nitrobenzaldehyde dehydrogenase is the only enzyme that has been partially purified from strain TW3⁷⁷ and only 4-nitrobenzoate nitroreductase and 4-hydroxylaminobenzoate lyase from *C. acidovorans* NBA-10⁴⁴ have been purified from the 4-nitrobenzoate portion of the pathway. The limited set of gene sequences reveals that the nitrobenzoate degradation genes from *Pseudomonas* sp. YH102 and *P. putida* TW3 share high sequence identity with each other and lesser identity with *R. pickettii* YH105. There are no other closely related genes in the GenBank database. This has been interpreted to indicate that the nitrobenzoate genes are ancient in origin and have diverged in the current host organisms^{73, 173}. The similarity of function of hydroxylaminobenzoate lyase to

hydroxylaminobenzene mutase thus appears to be a case of convergence. In contrast, the initial oxidation of 4-nitrotoluene to 4-nitrobenzoate is encoded by genes that appear to be derived from the TOL pathway. There is a high degree of sequence identity and identical gene order between the *xylWCMAB* genes of the TOL pathway and the *ntn(U)WCMAB* genes of the 4-nitrotoluene pathway⁷⁷. One gene that appears to be unique to the 4-nitrotoluene pathway is *ntnD*, the gene that encodes 4-nitrobenzyl alcohol dehydrogenase⁷⁶. The gene is located downstream of the rest of the *ntn* gene cluster and is not related to *xylB* of the TOL pathway. The *ntn* genes have a low G+C content compared to the surrounding sequences and transposase-like genes are located nearby. The authors propose that the *ntn* cluster was acquired by TW3 through a transposition event, during which the *xylB* gene became inactivated. Then *ntnD* was recruited from elsewhere to compensate for the inactivation of *xylB*.

2.1.3. Reduction to the Amine

Despite the widespread occurrence of nonspecific nitroreductases that can reduce nitro groups to the amine, few productive pathways that incorporate the strategy have been reported. Aerobic organisms reported to use the strategy are limited to *Pseudomonas* sp. JX165 for the degradation of NB¹⁶⁰ and *Arthrobacter protophormiae* RKJ100 for degradation of 2-nitrobenzoate²⁰. The evidence for both pathways is still preliminary.

Anaerobic transformation of nitroaromatic compounds accounts for most reports of reduction to the amine. Numerous recent reviews discuss much of the anaerobic reduction literature with particular regard to transformation of TNT^{3, 23, 34, 91, 128}. TNT has received so much attention because it has been the most widely used explosive in the world, and massive contamination persists at numerous manufacturing sites. Both TNT and its reduced transformation products are highly toxic^{42, 68, 113, 140}. No bacterium has been identified that can use TNT as a carbon source, although there are instances where TNT can serve as a nitrogen source^{12, 36, 38, 159}, or as the terminal electron acceptor³⁵.

Over the last 30 years there have been numerous reports of TNT transformation involving sequential reduction of the nitro groups to form aminodinitrotoluenes, followed by diaminonitrotoluenes, and at very low redox potentials, triaminotoluene¹²⁸. Each reduction step involves formation of nitroso- and hydroxylamino-intermediates. The partially reduced intermediates are highly reactive and can condense to form a variety of azoxy compounds^{2, 163} or bind to humic acids, cells, and soil^{1, 2, 25}. Recently, Hughes and coworkers found that *Clostridium acetylbutylicum* transforms TNT to a variety of hydroxylamino compounds rather than to the amine⁷¹. At least one product, a phenolic amine, is the result of a Bamberger rearrangement similar to the reaction catalyzed by hydroxylaminobenzene mutase, but the product is a *p*-aminophenol rather than an *o*-aminophenol⁷⁰. A carbon monoxide dehydrogenase in *Clostridium*

*thermoacetivum*⁶⁹ and an Fe-only hydrogenase in *C. acetylbutylicum*¹⁶¹ catalyze reduction of TNT to hydroxylamino compounds. Prolonged incubation of the resulting hydroxylamino compounds with the purified enzymes results in the rearrangement product previously discovered in whole cell systems. The identical rearrangement is also achieved abiotically via acid catalysis so that the formation of the phenolic amine cannot as yet be attributed to hydrogenase activity with any certainty.

Pseudomonas sp. JLR11 was originally isolated because it could use TNT as a sole nitrogen source when glucose was supplied under anaerobic conditions³⁶. What made it unusual was the high percentage (85%) of nitrogen that was assimilated by the cells. Although a variety of oxidized as well as reduced intermediates were identified in culture fluids, the aromatic ring remained intact. The authors proposed that nitrite was released from the oxidized intermediates, but not from any of the reduced products. The authors subsequently discovered that the reduced intermediates were produced because the bacterium also uses TNT as an electron acceptor for energy production under anaerobic conditions³⁵ (Ramos *et al.*, Chapter 8, this volume). In a system in which TNT was the only electron acceptor, ATP synthesis was coupled to proton extrusion and TNT reduction. The ability to use TNT as an electron acceptor for energy production might not be unique to strain JLR11, but the ability has not been rigorously demonstrated for any other bacterium.

2.2. Reduction of the Ring: Hydride–Meisenheimer Complex Formation

The presence of multiple nitro groups on an aromatic ring makes the ring so electron deficient that it is susceptible to reduction^{91, 132}. 2,4-Dinitrophenol^{92–94}, 2,4,6-trinitrophenol (picric acid)^{8, 94, 133}, and TNT^{38, 114, 158, 159} can all be reduced via an enzyme-dependent transfer of a hydride ion to form a non-aromatic hydride–Meisenheimer complex. Early reports speculated that the Meisenheimer complexes of picric acid and TNT could spontaneously eliminate nitrite and rearomatize to form dinitrophenols and DNT; compounds known to be biodegradable. But a general mechanism for removal of nitro groups from trinitroaromatic compounds has not materialized. Whereas picric acid and 2,4-dinitrophenol are mineralized via pathways that involve a series of hydride ion transfers, a mixed picture of Meisenheimer complex involvement in TNT transformation has emerged⁶⁶. Although there are very early reports of unidentified pseudomonads that degrade picric acid, no details are known beyond the release of nitrite⁷⁸. Therefore, because most of the work on picric acid degradation has been done with various *Nocardioides* and *Rhodococcus* strains, picric acid and 2,4-dinitrophenol degradation will not be discussed.

Hydride–Meisenheimer complexes of TNT have been reported for a number of bacteria, but significant release of nitrite has only been reported for *Pseudomonas* sp. clone A⁴⁷, *P. fluorescens* I-C¹¹⁴, and *E. cloacae* PB2³⁸. Presumably the enzymes involved in hydride–Meisenheimer complex formation from TNT are not related to those involved in picric acid degradation because the gram-negative bacteria lack the F₄₂₀-dependent enzymes responsible for picric acid catabolism⁶⁷. Both the *Pseudomonas* strains catalyzed reduction of the nitro groups as well as the ring of TNT, whereas the *Enterobacter* converted TNT only to hydride–Meisenheimer complex intermediates. To date, only the *Enterobacter* is capable of growth with TNT as the sole source of nitrogen³⁸. *Pseudomonas* sp. clone A was reported to grow on TNT as the sole source of carbon, nitrogen, and energy³¹, but the finding has not been repeated¹⁵⁹. No information is available on whether *P. fluorescens* I-C. can use TNT as a growth substrate. As yet, the mechanism by which nitrite is released from TNT is not known. Pak *et al.*¹¹⁴ have proposed that an abiotic condensation reaction between a dihydride–Meisenheimer complex and a cation formed from 4-hydroxylamino-2,6-dinitrotoluene could result in a substituted biphenyl following the elimination of nitrite. The substituted biphenyl has not been reported as a TNT metabolite from any other system.

2.3. Monooxygenation of 4-Nitrophenol

Monooxygenation as a strategy for initial attack on nitroaromatic compounds is limited to 2- and 4-nitrophenol and their derivatives including parathion, 4-nitroanisole, and 4-chloro-2-nitrophenol (Figure 5). The key requirement is for a hydroxyl group *ortho* or *para* to a nitro group. The ability to degrade such compounds, however, is globally distributed both geographically and throughout the bacterial taxa, and numerous examples of 4-nitrophenol- and parathion-degrading bacteria are found in the literature. Table 1 lists only those studies in which the pathway has been determined. Little has been added to our understanding of monooxygenation of nitrophenols since the publication of several recent reviews and the reader is referred to those papers for detailed discussions^{82, 143, 144, 173}.

The ester linkages of parathion, substituted parathions, and 4-nitroanisole are cleaved prior to monooxygenation in the degradation pathways (Figure 5). Monooxygenation reactions can add hydroxyl groups at the 2 or 3 carbon positions of 4-nitrophenol to form 4-nitrocatechol or 4-nitroresorcinol. The nitrophenol, nitroresorcinol, or nitrocatechols are all substrates for the flavoprotein monooxygenases that catalyze the release of the nitro group and the formation of a benzoquinone intermediate. The participation of the quinone intermediate has only been proven in the conversion of 4-methyl-5-nitrocatechol to 2-hydroxy-5-methylquinone during degradation of 2,4-DNT. Benzoquinone

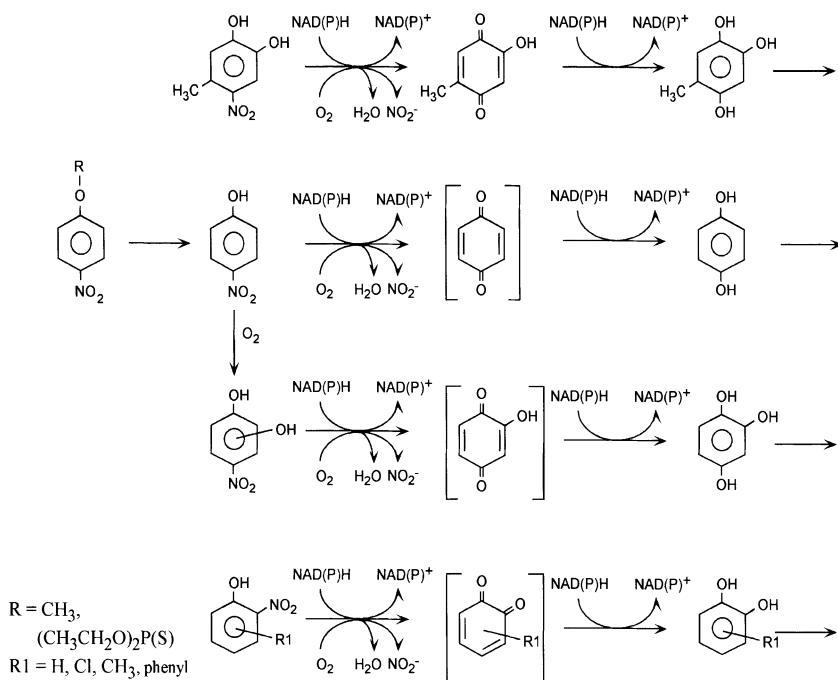


Figure 5. Monoxygenase attack on nitrophenols.

intermediates in the 2- and 4-nitrophenol pathways are removed too rapidly, both abiotically and enzymatically, for purification and identification. The benzoquinone intermediates in those pathways have been inferred from the occasional detection of traces of the compounds and from the requirement for 2 mol of NADPH to convert nitrophenol to hydroquinone or catechol. Flavoprotein monoxygenases catalyze the initial attack on nitrophenol and methylnitrocatechol, yet despite the similarity of the initial reactions, the enzymes are unlikely to be closely related. The genes that encode methylnitrocatechol monoxygenases have sequences that are most closely related to antibiotic synthesis monoxygenases, yet the overall operon organization is more closely related to those for phenol and monochlorophenol oxygenase degradation⁸². The 4-nitrophenol monoxygenase from *Pseudomonas* sp. ENV2030 is most closely related to 3-hydroxyphenylpropionate monoxygenases¹⁷³.

The benzoquinone intermediates are reduced accompanied by rearomatization to hydroquinone or trihydroxybenzene, catechol, or trihydroxytoluene, if the parent compounds are 4-nitrophenol, 2-nitrophenol, or 4-methyl-5-nitrocatechol, respectively. None of the quinone reductases have been purified. Dioxygenase enzymes catalyze ring fission of the hydroxylated intermediates and all the pathways converge on β -ketoadipate.

2.4. Initial Dioxygenation of Nitroaromatic Compounds

Dioxygenation is the classic mechanism for bacterial degradation of aromatic compounds^{119a}, yet until the early 1990s no nitroaromatic compounds were known to be degraded via initial dioxygenation reactions. Since that time dioxygenation has proven to be the most widely used mechanism for initiating mineralization of nonpolar nitroaromatic compounds (Figure 1). Briefly, degradation is initiated by attack at the nitro group farthest from any substituent (Figure 6). Two hydroxyl groups are inserted on the ring to form nitrohydrodiols. The nitrohydrodiols spontaneously rearomatize with the release of nitrite to yield catechols. Although the reactions involved are analogous to those of the toluene dioxygenase pathway⁴¹, nitroarene dioxygenases are only distantly related to toluene dioxygenase. The proposed nitrohydrodiol intermediates have not been detected, nor does the elimination of nitrite require an enzyme with function analogous to toluene dihydrodiol dehydrogenase. The range of growth substrates for bacteria that possess nitroarene dioxygenases is

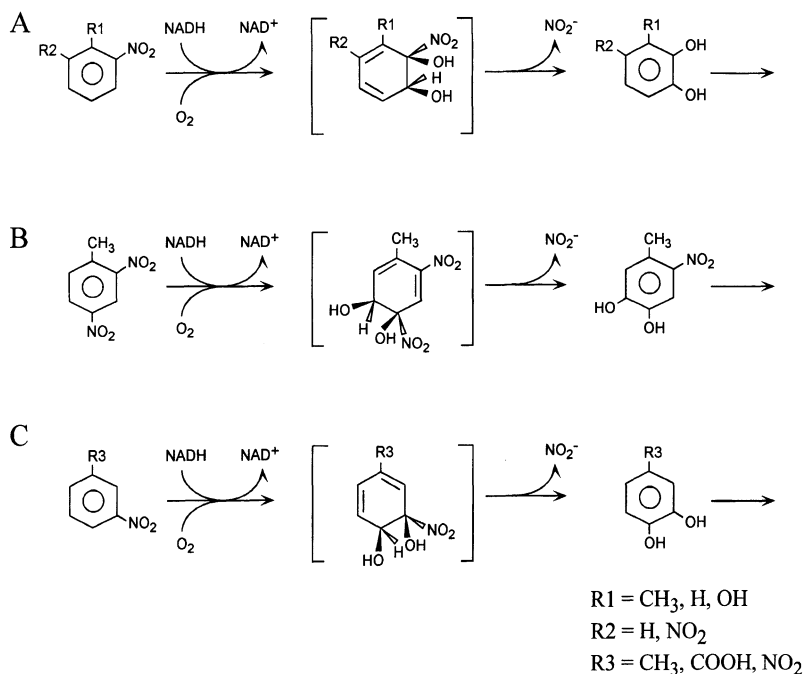


Figure 6. Dioxygenase catalyzed initial reactions with A. NB, 2-nitrotoluene, 2,6-dinitrotoluene, and 2,6-dinitrophenol; B. 2,4-dinitrotoluene; and C. 3-nitrobenzoate, 3-nitrotoluene, and 1,3-dinitrobenzene.

narrow, but the nitroarene dioxygenases themselves have very broad substrate ranges⁹⁵.

Most of our understanding of the dioxygenase initiated pathways comes from studies of the degradation of 2,4-DNT by *Burkholderia* sp. DNT and *B. cepacia* R34, 2-nitrotoluene (2-NT) by *Acidovorax* sp. JS42, and NB by *Comamonas* sp. JS765. Many of the genes involved in the pathways have been cloned and sequenced, enzymes have been purified or partially purified, and the regulatory genes have been identified. The first indication that initial steps in nitroaromatic and naphthalene degradation pathways are related came from sequence analysis of the genes for DNT dioxygenase in *Burkholderia* sp. DNT¹⁵⁰. All nitroarene dioxygenases are highly related and can be distinguished as a group from naphthalene dioxygenases⁸². The structural and regulatory genes that encode nitroarene dioxygenases form a cluster that is closely related to the *nag* genes that encode the upper pathway for naphthalene degradation via gentisate found in *Ralstonia* sp. U2^{39, 172} and is more distantly related to the *nah* genes that encode naphthalene dioxygenase in the archetypal pathway for naphthalene degradation via catechol. Recent reviews^{82, 117} discuss the details of the genes involved in the degradation pathways and the current understanding of the evolutionary origins of the pathways.

The *nbzR* gene from *Comamonas* sp. JS765 and the *ntdR* gene from *Acidovorax* sp. JS42 encode identical LysR-type regulatory proteins⁹⁶. The proteins differ from the NagR regulatory protein by only five amino acids, but the NagR regulatory protein responds only to salicylate, whereas the NtdR and NbzR proteins respond to NB, nitrotoluenes, DNT, and aminodinitrotoluenes as well as salicylate and anthranilate. The studies demonstrated that the nitroaromatic compounds themselves serve as the effectors in the dioxygenase pathways because no responses were obtained with the known pathway intermediates catechol and 3-methylcatechol. It should also be noted that while the regulatory proteins respond to a wide variety of effector compounds, most of the effectors do not serve as growth substrates for the various strains. Bacteria generally are not able to degrade multiple nitroaromatic compounds even though nitroaromatic compounds are normally present as complex mixtures. It seems likely that the inability of bacteria to degrade a broad range of nitroaromatic compounds lies in limitations of the lower pathways and not with the initial dioxygenases or their regulation. It appears that the two major challenges for the evolution of dioxygenase initiated nitroaromatic catabolic pathways is to first, match the correct lower pathway to the product of the initial dioxygenation reaction, and second, regulate the lower pathway to avoid misrouting and the formation of dead-end and toxic products.

Comamonas sp. JS765, *Acidovorax* JS42, and *Burkholderia* sp. JS52 catalyze the oxidation of NB, 2-nitrotoluene, and 3-nitrobenzoate, to catechol, 3-methylcatechol, and protocatechuate, respectively. The three dihydroxy

compounds are common intermediates in aromatic metabolism and feed into central metabolic pathways.

The pathways for dinitroaromatic compound degradation are more complex because the catechol intermediates still have a nitro group on the ring. Two different strategies have evolved to deal with the nitroaromatic intermediates: direct ring cleavage with a nitro group still on the ring, or removal of the nitro group before ring cleavage (Figure 7). The catechol intermediates in the 2,6-DNT pathway found in *B. cepacia* JS850¹⁰⁸ and in the 2,6-dinitrophenol pathway found in *R. eutrophus* JMP134 (2,6-DNP)³³, are directly cleaved, and in both pathways the second nitro group is released as nitrite via unknown subsequent reactions. The number of examples is too small to generalize that the nitrocatechol intermediates of all 2,6-dinitroaromatic pathways are cleaved with a nitro group still attached to the ring. Cleavage of 3-methyl-4-nitrocatechol is extradiol in the 2,6-DNT degradation pathway and the ring cleavage product, 2-hydroxy-5-nitro-6-oxohepta-2,4-dienoic acid is enzymatically hydrolyzed to 2-hydroxy-5-nitropenta-2,4-dienoic acid¹⁰⁸. In contrast, preliminary evidence suggests that nitropyrogallol probably undergoes intradiol cleavage in the 2,6-DNP pathway³³. A transient intermediate of cleavage of nitropyrogallol by cell extracts of 2,6-DNP-grown cultures of strain JMP134 was proposed to be 2-hydroxy-5-nitro-muconic acid based on its conversion to 2-hydroxy-5-nitropenta-2,4-dienoic acid.

In the 2,4-DNT degradation pathway, the second nitro group is removed prior to ring cleavage. Detailed studies that include enzyme purification and cloning and sequencing of the genes have been carried out with *Burkholderia* sp. DNT and *B. cepacia* R34^{48, 49, 51, 80, 81, 116, 118, 148, 150, 151}. As mentioned in the previous section, 4-methyl-5-nitrocatechol is not cleaved directly, but undergoes a monooxygenation reaction to form 2-hydroxy-5-methylquinone with the loss of the second nitro group as nitrite. The quinone is reduced to 2,4,5-trihydroxytoluene which is cleaved by an extradiol ring cleavage dioxygenase to 2,4-dihydroxy-5-methyl-6-oxo-2,4-hexadienoic acid^{48, 80}. The ring fission product is presumed to undergo tautomerization to the keto form which is cleaved to yield pyruvic acid and methylmalonic acid semialdehyde. The same enzyme, DntG, catalyzed both the tautomerization and hydrolysis reactions⁸¹.

The recent completion of analysis of the reactions and genes involved in the pathway for degradation of 2,4-DNT by *B. cepacia* R34⁸¹ has extended our understanding of the evolution of nitroaromatic pathways. The 2,4-DNT pathway in strain R34 appears to have been constructed by recruitment of three separate modules for the initial dioxygenase, the monooxygenase, and the ring-fission dioxygenase⁸². As discussed above, both the regulatory genes and the structural genes for the initial dioxygenases are derived from the naphthalene degradation pathway in which salicylate is converted to gentisate by a hydroxylase. Whereas NagR from *Ralstonia* sp. U2 only responds to salicylate,

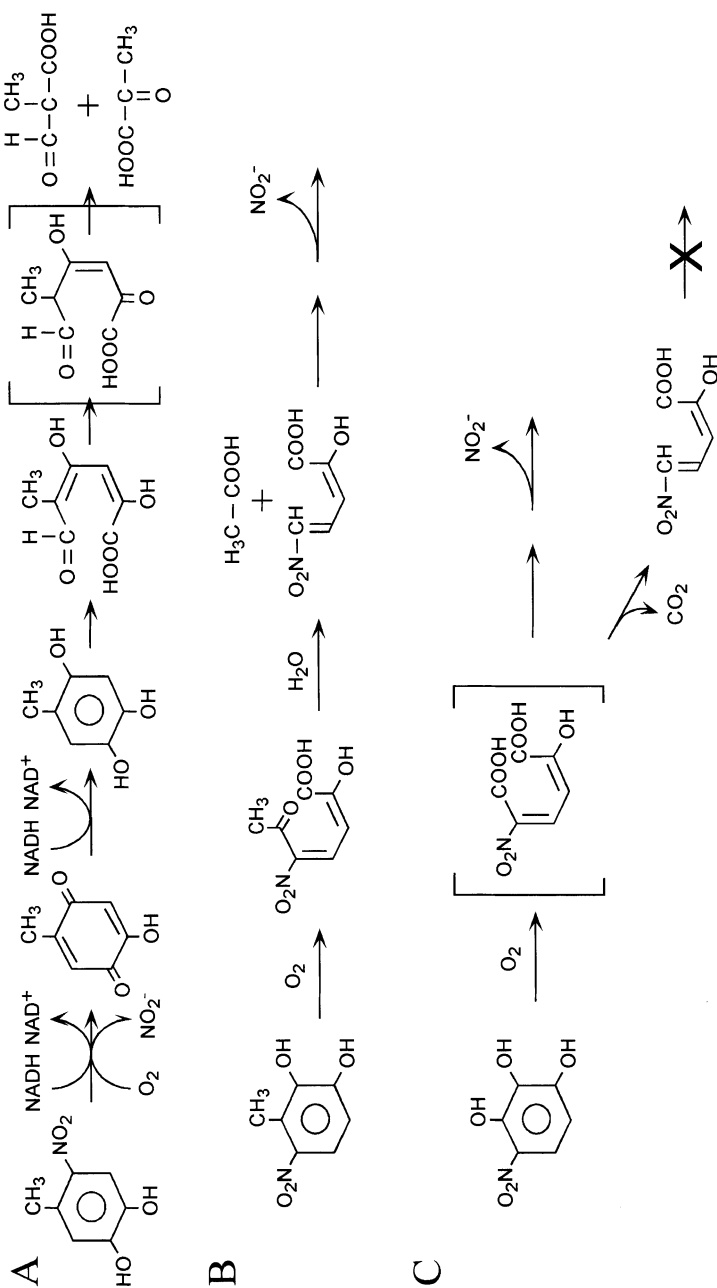


Figure 7. Cleavage of intermediates of A. 2,4-DNT, B. 2,6-DNT, and C. 2,6-DNP degradation pathways.

preliminary studies with DntR indicate that the effector range is broad (Parales, personal communication). Sequences that are remnants of salicylate hydroxylase genes from the *nag* pathway have been found in all nitroarene dioxygenase operons analyzed to date⁸². It seems likely that the genes are being eliminated as more efficient and compact operons evolve^{81, 82}.

The methylnitrocatechol monooxygenases distinguish 2,4-DNT degradation pathways from all other nitroarene dioxygenase initiated pathways, yet the monooxygenases from *Burkholderia* sp. DNT and *B. cepacia* R34 share only 53% identity⁸¹. The trihydroxytoluene dioxygenase genes from the two strains share only 60% identity⁸⁰. The regulation of the methylnitrocatechol monooxygenase and the trihydroxytoluene dioxygenase genes has not yet been worked out. A major determinant of how well a strain grows with 2,4-DNT is the coordination of the expression of the three oxygenase genes. Thus, slow growing strains such as DNT and R34 exhibit lag periods before the methylnitrocatechol monooxygenase and trihydroxytoluene dioxygenases are expressed and full induction of the pathway can take up to 4–5 days. Fast growing strains such as *B. cepacia* JS872 are fully induced within several hours after exposure to 2,4-DNT¹⁰⁸.

In general, the genes for the nitroarene dioxygenases and their regulators have spread by horizontal gene transfer in the recent past⁸². But the acquisition of the remaining elements of the degradation pathways has not been a uniform process. Evolution has taken advantage of existing catabolic pathways for the simpler nitroaromatic compounds, but more complex changes had to occur before dinitroaromatic compounds could be utilized as sole growth substrates. It seems likely that the pathways evolved in the past century in response to the release of synthetic chemicals in the biosphere. If so, the greater complexity of the changes involved might be measured by the time between the discovery of bacteria that could degrade mononitrobenzoates and mononitrophenols and those that degrade DNT, a period of about 30 years.

3. APPLICATIONS

One of the major contributions of the basic work described above is the understanding that natural bacteria are degrading nitroaromatic compounds at a wide range of contaminated sites. Understanding of the pathways and strategies used by bacteria to degrade nitroaromatic compounds will enhance our ability to predict the behavior of such bacteria in the environment and can form the basis for specific cleanup strategies, including natural attenuation. Elucidation of the genes responsible for the catalytic abilities can provide the basis for metabolic engineering for degradation of recalcitrant compounds such as TNT.

Biodegradation, including natural attenuation, is now being applied at military and industrial sites that are contaminated with nitroaromatic compounds^{24, 79}. More recently, the novel enzymes unique to nitroaromatic degradation pathways have found potential biocatalytic applications for the production of chemicals that are difficult to synthesize through conventional means. The hydroxylaminobenzene mutase from *P. pseudoalcaligenes* JS45 can be used to make a variety of aminophenols¹⁰⁵. The mutase coupled with the aminophenol ring fission dioxygenase can simplify the production of novel picolinic acids⁶³. Nitroarene dioxygenases add to the arsenal of dihydroxylating dioxygenases that are available for biocatalysis of unusual catechols and chiral compounds^{13, 40, 87, 100}.

It is clear that a wide range of bacteria have evolved the ability to take advantage of most of the industrially important nitroaromatic compounds. The biochemical strategies they have assembled are fairly well understood. In contrast, the regulation of the pathways, their evolution, and how they relate to microbial interactions in natural ecosystems have not been explored. Although there are a few natural nitroaromatic compounds, it seems likely that the chemicals discussed here have only been in the biosphere for the past century. Thus, the pathways appear to have evolved recently and are still evolving as indicated by their inefficient regulation and the presence of extra genetic material indicative of recent horizontal gene transfer. Recent advances in sequencing capabilities will allow rapid advances in understanding how microbes ensure the continued participation of nitroaromatic compounds in the global carbon cycle.

REFERENCES

1. Achtnich, C., Sieglén, U., Knackmuss, H.-J., and Lenke, H., 1999, Irreversible binding of biologically reduced 2,4,6-trinitrotoluene to soil. *Environ. Toxicol. Chem.*, 18:2416–2423.
2. Ahmad, F. and Hughes, J.B., 2002, Reactivity of partially reduced arylhydroxylamino and nitrosoarene metabolites of 2,4,6-trinitrotoluene (TNT) toward biomass and humic acids. *Environ. Sci. Technol.*, 36:4370–4381.
3. Ahmad, F. and Hughes, J.B., 2000, Anaerobic transformation of TNT by *Clostridium*, p. 185–212. In J.C. Spain, J.B. Hughes, and H.-J. Knackmuss (eds), *Biodegradation of Nitroaromatic Compounds and Explosives*. Lewis Publishers, Boca Raton.
4. Ali-Sadat, S., Mohan, K.S., and Walia, S.K., 1995, A novel pathway for the biodegradation of 3-nitrotoluene in *Pseudomonas putida*. *FEMS Microbiol. Ecol.*, 17:169–176.
5. An, D., Gibson, D.T., and Spain, J.C., 1994, Oxidative release of nitrite from 2-nitrotoluene by a three-component enzyme system from *Pseudomonas* sp. strain JS42. *J. Bacteriol.*, 176:7462–7467.
6. Aoki, K., Takenaka, S., Murakami, S., and Shinke, R., 1997, Partial purification and characterization of a bacterial dioxygenase that catalyzes the ring fission of 2-aminophenol. *Microbiol. Res.*, 152:33–38.
7. Barbieri, P., Arengi, F.L.G., Bertoni, G., Bolognese, F., and Galli, E., 2001, Evolution of catabolic pathways and metabolic versatility in *Pseudomonas stutzeri* OX1. *Antonie van Leeuwenhoek*, 79:135–140.

8. Behrend, C. and Heesche-Wagner, K., 1999, Formation of Hydride-Meisenheimer complexes of picric acid (2,4,6-trinitrophenol) and 2,4-dinitrophenol during mineralization of picric acid by *Nocardioides* sp. strain CB 22-2. *Appl. Environ. Microbiol.*, 65:1372–1377.
9. Bhushan, B., Chauhan, A., Samanta, S.K., and Jain, R.K., 2000, Kinetics of biodegradation of *p*-nitrophenol by different bacteria. *Biochem. Biophys. Res. Comm.*, 274:626–630.
10. Blasco, R., Moore, E., Wray, V., Pieper, D., Timmis, K., and Castillo, F., 1999, 3-Nitroadipate, a metabolic intermediate for mineralization of 2,4-dinitrophenol by a new strain of a *Rhodococcus* species. *J. Bacteriol.*, 181:149–152.
11. Blotevogel, K.-H. and Gorontzy, T., 2000, Microbial degradation of compounds with nitro functions. In H.-J. Rehm, G. Reed, A. Pühler, and P. Stadler (eds), *Biotechnology*, 2nd ed., vol. 11, pp. 273–302. Wiley-VCH, Weinheim, Germany.
12. Boopathy, R. and Kulpa, C.F., 1992, Trinitrotoluene (TNT) as a sole nitrogen source for a sulfate-reducing bacterium *Desulfovibrio* sp. (B strain) isolated from an anaerobic digester. *Curr. Microbiol.*, 25:235–241.
13. Boyd, D.R., Sharma, N.D., and Allen, C.C.R., 2001, Aromatic dioxygenases: Molecular biocatalysis and applications. *Curr. Opin. Biotechnol.*, 12:564–573.
14. Bruhn, C., Bayly, R.C., and Knackmuss, H.-J., 1988, The in vivo construction of 4-chloro-2-nitrophenol assimilatory bacteria. *Arch. Microbiol.*, 150:171–177.
15. Cain, R.B., 1966, Utilization of anthranilic and nitrobenzoic acids by *Nocardia opaca* and a flavobacterium. *J. Gen. Microbiol.*, 42:219–235.
16. Cartwright, N.J. and Cain, R.B., 1959, Bacterial degradation of the nitrobenzoic acids. 2. Reduction of the nitro group. *Biochem. J.*, 73:305–314.
17. Cassidy, M.B., Lee, H., Trevors, J.T., and Zablotowicz, R.B., 1999, Chlorophenol and nitrophenol metabolism by *Sphingomonas* sp UG30. *J. Ind. Microbiol. Biotechnol.*, 23:232–241.
18. Cerniglia, C.E. and Somerville, C.C., 1995, Reductive metabolism of nitroaromatic and nitropolycyclic aromatic hydrocarbons. In J.C. Spain (ed.), *Biodegradation of Nitroaromatic Compounds*, pp. 99–115. Plenum Publishing Corp., New York.
19. Chauhan, A. and Jain, R.K., 2000, Degradation of *o*-nitrobenzoate via anthranilic acid (*o*-aminobenzoate) by *Arthrobacter protophormiae*: A plasmid-encoded new pathway. *Biochem. Biophys. Res. Comm.*, 267:236–244.
20. Chauhan, A., Samanta, S.K., and Jain, R.K., 2000, Degradation of 4-nitrocatechol by *Burkholderia cepacia*: A plasmid-encoded novel pathway. *J. Appl. Microbiol.*, 88:764–772.
21. Copley, S.D., 2003, Enzymes with extra talents: Moonlighting functions and catalytic promiscuity. *Curr. Opin. Chem. Biol.*, 7:265–272.
22. Corbett, M.D. and Corbett, B., 1995, Bioorganic chemistry of the arylhydroxylamine and nitrosoarene functional groups. In J.C. Spain (ed.), *Biodegradation of Nitroaromatic Compounds*, pp. 151–182. Plenum Publishing Corp., New York.
23. Crawford, R.L., 1995, Biodegradation of nitrated munition compounds and herbicides by obligately anaerobic bacteria. In J.C. Spain (ed.), *Biodegradation of Nitroaromatic Compounds*, pp. 87–98. Plenum Publishing Corp., New York.
24. Cuffin, S.M., Lafferty, P.M., Taylor, P.N., Spain, J.C., Nishino, S.F., and Williams, K.A., 2001, Bioremediation of dinitrotoluene isomers in the unsaturated/saturated zone, abstr. *Poster Session B1, Sixth International In Situ and On-Site Bioremediation Symposium*. San Diego, California.
25. Daun, G., Lenke, H., Reuss, M., and Knackmuss, H.-J., 1998, Biological treatment of TNT-contaminated soil. 1. Anaerobic cometabolic reduction and interaction of TNT and metabolites with soil components. *Environ. Sci. Technol.*, 32:1956–1963.
26. Davis, J.K., He, Z., Somerville, C.C., and Spain, J.C., 1999, Genetic and biochemical comparison of 2-aminophenol-1,6-dioxygenase of *Pseudomonas pseudoalcaligenes* JS45 to *meta*-cleavage dioxygenases: Divergent evolution of 2-aminophenol *meta*-cleavage pathway. *Arch. Microbiol.*, 172:330–339.

27. Davis, J.K., Paoli, G.C., He, Z., Nadeau, L.J., Somerville, C.C., and Spain, J.C., 2000, Sequence analysis and initial characterization of two isozymes of hydroxylaminobenzene mutase from *Pseudomonas pseudoalcaligenes* JS45. *Appl. Environ. Microbiol.*, 66:2965–2971.
28. de la Cruz, F. and Davies, J., 2000, Horizontal gene transfer and the origin of species: Lessons from bacteria. *Trends Microbiol.*, 8:128–133.
29. Delgado, A., Wubbolts, M.G., Abril, M.-A., and Ramos, J.L., 1992, Nitroaromatics are substrates for the TOL plasmid upper-pathway enzymes. *Appl. Environ. Microbiol.*, 58:415–417.
30. Dickel, O. and Knackmuss, H.-J., 1991, Catabolism of 1,3-dinitrobenzene by *Rhodococcus* sp. QT-1. *Arch. Microbiol.*, 157:76–79.
31. Duque, E., Haidour, A., Godoy, F., and Ramos, J.L., 1993, Construction of a *Pseudomonas* hybrid strain that mineralizes 2,4,6-trinitrotoluene. *J. Bacteriol.*, 175:2278–2283.
32. Ebert, S., Rieger, P.-G., and Knackmuss, H.-J., 1999, Function of coenzyme F₄₂₀ in aerobic catabolism of 2,4,6-trinitrophenol and 2,4-dinitrophenol by *Nocardioideis simplex* FJ2-1A. *J. Bacteriol.*, 181:2669–2674.
33. Ecker, S., Widmann, T., Lenke, H., Dickel, O., Fischer, P., Bruhn, C., and Knackmuss, H.-J., 1992, Catabolism of 2,6-dinitrophenol by *Alcaligenes eutrophus* JMP134 and JMP222. *Arch. Microbiol.*, 158:149–154.
34. Esteve-Nunez, A., Caballero, A., and Ramos, J.L., 2001, Biological degradation of 2,4,6-trinitrotoluene. *Microbiol. Mol. Biol. Rev.*, 65:335–352.
35. Esteve-Núñez, A., Lucchesi, G., Philipp, B., Schink, B., and Ramos, J.L., 2000, Respiration of 2,4,6-trinitrotoluene by *Pseudomonas* sp. strain JLR11. *J. Bacteriol.*, 182:1352–1355.
36. Esteve-Núñez, A. and Ramos, J.L., 1998, Metabolism of 2,4,6-trinitrotoluene by *Pseudomonas* sp. JLR11. *Environ. Sci. Technol.*, 32:3802–3808.
37. Fiorella, P.D. and Spain, J.C., 1997, Transformation of 2,4,6-trinitrotoluene by *Pseudomonas pseudoalcaligenes* JS52. *Appl. Environ. Microbiol.*, 63:2007–2015.
38. French, C.E., Nicklin, S., and Bruce, N.C., 1998, Aerobic degradation of 2,4,6-trinitrotoluene by *Enterobacter cloacae* PB2 and by pentaerythritol tetranitrate reductase. *Appl. Environ. Microbiol.*, 64:2864–2868.
39. Fuenmayer, S.L., Wild, M., Boyes, A.L., and Williams, P.A., 1998, A gene cluster encoding steps in conversion of naphthalene to gentisate in *Pseudomonas* sp. strain U2. *J. Bacteriol.*, 180:2522–2530.
40. Gibson, D.T. and Parales, R.E., 2000, Aromatic hydrocarbon dioxygenases in environmental biotechnology. *Curr. Opin. Biotechnol.*, 11:236–243.
41. Gibson, D.T. and Subramanian, V., 1984, Microbial degradation of aromatic hydrocarbons. In D.T. Gibson (ed.), *Microbial Degradation of Organic Compounds*, pp. 181–252. Marcel Dekker, Inc., New York.
42. Gong, P., Siciliano, S.D., Greer, C.W., Paquet, L., Hawari, J., and Sunahara, G., 1999, Effects and bioavailability of 2,4,6-trinitrotoluene in spiked and field-contaminated soils to indigenous microorganisms. *Environ. Toxicol. Chem.*, 18:2681–2688.
43. Goodall, J.L., Thomas, S.M., Spain, J.C., and Peretti, S.W., 1998, Operation of mixed-culture immobilized cell reactors for the metabolism of *meta*- and *para*-nitrobenzoate by *Comamonas* sp. JS46 and *Comamonas* sp. JS47. *Biotechnol. Bioeng.*, 59:21–27.
44. Groenewegen, P.E.J., Breeuwer, P., van Helvoort, J.M.L.M., Langenhoff, A.A.M., de Vries, F.P., and de Bont, J.A.M., 1992, Novel degradative pathway of 4-nitrobenzoate in *Comamonas acidovorans* NBA-10. *J. Gen. Microbiol.*, 138:1599–1605.
45. Groenewegen, P.E.J. and de Bont, J.A.M., 1992, Degradation of 4-nitrobenzoate via 4-hydroxylaminobenzoate and 3,4-dihydroxybenzoate in *Comamonas acidovorans* NBA-10. *Arch. Microbiol.*, 158:381–386.
46. Gundersen, K. and Jensen, H.L., 1956, A soil bacterium decomposing organic nitro-compounds. *Acta Agr. Scand.*, 6:100–114.

47. Haïdour, A. and Ramos, J.L., 1996, Identification of products resulting from the biological reduction of 2,4,6-trinitrotoluene, 2,4-dinitrotoluene, and 2,6-dinitrotoluene by *Pseudomonas* sp. *Environ. Sci. Technol.*, 30:2365–2370.
48. Haigler, B.E., Johnson, G.R., Suen, W.-C., and Spain, J.C., 1999, Biochemical and genetic evidence for meta-ring cleavage of 2,4,5-trihydroxytoluene in *Burkholderia* sp. strain DNT. *J. Bacteriol.*, 181:3965–3972.
49. Haigler, B.E., Nishino, S.F., and Spain, J.C., 1994, Biodegradation of 4-methyl-5-nitrocatechol by *Pseudomonas* sp. strain DNT. *J. Bacteriol.*, 176:3433–3437.
50. Haigler, B.E. and Spain, J.C., 1993, Biodegradation of 4-nitrotoluene by *Pseudomonas* sp. strain 4NT. *Appl. Environ. Microbiol.*, 59:2239–2243.
51. Haigler, B.E., Suen, W.-C., and Spain, J.C., 1996, Purification and sequence analysis of 4-methyl-5-nitrocatechol oxygenase from *Burkholderia* sp. strain DNT. *J. Bacteriol.*, 178:6019–6024.
52. Haigler, B.E., Wallace, W.H., and Spain, J.C., 1994, Biodegradation of 2-nitrotoluene by *Pseudomonas* sp. Strain JS42. *Appl. Environ. Microbiol.*, 60:3466–3469.
53. Hamdi, Y.A., and Tewfik, M.S., 1970, Degradation of 3,5-dinitro-*o*-cresol by *Rhizobium* and *Azotobacter* spp. *Soil Biol. Biochem.*, 2:163–166.
54. Hammill, T.B. and Crawford, R.L., 1996, Degradation of 2-sec-butyl-4,6-dinitrophenol (dinoseb) by *Clostridium bifermentans* KMR-1. *Appl. Environ. Microbiol.*, 62:1842–1846.
55. Harayama, S. and Rekik, M., 1993, Comparison of the nucleotide sequences of the meta-cleavage pathway genes of TOL plasmid pWW0 from *Pseudomonas putida* with other meta-cleavage genes suggests that both single and multiple nucleotide substitutions contribute to enzyme evolution. *Mol. Gen. Genet.*, 239:81–89.
56. Harayama, S., Rekik, M., Ngai, K.-L., and Ornston, L.N., 1989, Physically associated enzymes produce and metabolize 2-hydroxy-2,4-dienoate, a chemically unstable intermediate formed in catechol metabolism via meta cleavage in *Pseudomonas putida*. *J. Bacteriol.*, 171:6251–6258.
57. Haro, M.-A. and de Lorenzo, V., 2001, Metabolic engineering of bacteria for environmental applications: Construction of *Pseudomonas* strains for biodegradation of 2-chlorotoluene. *J. Biotechnol.*, 85:103–113.
58. He, Z., Davis, J.K., and Spain, J.C., 1998, Purification, characterization, and sequence analysis of 2-aminomuconic 6-semialdehyde dehydrogenase from *Pseudomonas pseudoalcaligenes* JS45. *J. Bacteriol.*, 180:4591–4595.
59. He, Z., Nadeau, L.J., and Spain, J.C., 2000, Characterization of hydroxylaminobenzene mutase from pNBZ139 cloned from *Pseudomonas pseudoalcaligenes* JS45. A highly associated SDS-stable enzyme catalyzing an intramolecular transfer of hydroxy groups. *Eur. J. Biochem.*, 267:1110–1116.
60. He, Z. and Spain, J.C., 1997, Studies of the catabolic pathway of degradation of nitrobenzene by *Pseudomonas pseudoalcaligenes* JS45: Removal of the amino group from 2-aminomuconic semialdehyde. *Appl. Environ. Microbiol.*, 63:4839–4843.
61. He, Z. and Spain, J.C., 1998, A novel 2-aminomuconate deaminase in the nitrobenzene degradation pathway of *Pseudomonas pseudoalcaligenes* JS45. *J. Bacteriol.*, 180:2502–2506.
62. He, Z. and Spain, J.C., 1999, Comparison of the downstream pathways for degradation of nitrobenzene by *Pseudomonas pseudoalcaligenes* JS45 (2-aminophenol pathway) and by *Comamonas* sp. JS765 (catechol pathway). *Arch. Microbiol.*, 171:309–316.
63. He, Z. and Spain, J.C., 2000, One-step production of picolinic acids from 2-aminophenols catalyzed by 2-aminophenol 1,6-dioxygenase. *J. Ind. Microbiol. Biotechnol.*, 25:25–28.
64. He, Z. and Spain, J.C., 2000, Reactions involved in the lower pathway for degradation of 4-nitrotoluene by *Mycobacterium* strain HL 4-NT-1. *Appl. Environ. Microbiol.*, 66:2910–2911.
65. Heiss, G., Hofmann, K.W., Tractmann, N., Walters, D.M., Rouvière, P., and Knackmuss, H.-J., 2002, *npd* gene functions of *Rhodococcus (opacus) erythropolis* HL PM-1 in the initial steps of 2,4,6-trinitrophenol degradation. *Microbiology*, 148:799–806.

66. Heiss, G. and Knackmuss, H.-J., 2002, Bioelimination of trinitroaromatic compounds: Immobilization versus mineralization. *Curr. Opin. Microbiol.*, 5:282–287.
67. Heiss, G., Trachtmann, N., Abe, Y., Takeo, M., and Knackmuss, H.-J., 2003, Homologous *npdGI* genes in 2,4-dinitrophenol- and 4-nitrophenol-degrading *Rhodococcus* spp. *Appl. Environ. Microbiol.*, 69:2748–2754.
68. Honeycutt, M.E., Jarvis, A.S., and Mcfarland, V.A., 1996, Cytotoxicity and mutagenicity of 2,4,6-trinitrotoluene and its metabolites. *Ecotoxicol. Environ. Saf.*, 35:282–287.
69. Huang, S., Lindahl, P.A., Wang, C., Bennett, G.N., Rudolph, F.B., and Hughes, J.B., 2000, 2,4,6-Trinitrotoluene reduction by carbon monoxide dehydrogenase from *Clostridium thermoaceticum*. *Appl. Environ. Microbiol.*, 66:1474–1478.
70. Hughes, J.B., Wang, C., Yesland, K., Richardson, A., Bhadra, R., Bennett, G., and Rudolph, F., 1998, Bamberger rearrangement during TNT metabolism by *Clostridium acetobutylicum*. *Environ. Sci. Technol.*, 32:494–500.
71. Hughes, J.B., Wang, C.Y., Bhadra, R., Richardson, A., Bennett, G.N., and Rudolph, F.B., 1998, Reduction of 2,4,6-trinitrotoluene by *Clostridium acetobutylicum* through hydroxylamino-nitrotoluene intermediates. *Environ. Toxicol. Chem.*, 17:343–348.
72. Hughes, M.A., Baggs, M.J., al-Dulayymi, J.a., Baird, M.S., and Williams, P.A., 2002, Accumulation of 2-aminophenoxazin-3-one-7-carboxylate during growth of *Pseudomonas putida* TW3 on 4-nitro-substituted substrates requires 4-hydroxylaminobenzoate lyase (PnbB). *Appl. Environ. Microbiol.*, 68:4965–4970.
73. Hughes, M.A. and Williams, P.A., 2001, Cloning and characterization of the *pnb* genes, encoding enzymes for 4-nitrobenzoate catabolism in *Pseudomonas putida* TW3. *J. Bacteriol.*, 183:1225–1232.
74. Jain, R., Rivera, M.C., and Lake, J.A., 1999, Horizontal gene transfer among genomes: The complexity hypothesis. *Proc. Natl. Acad. Sci., USA*, 96:3801–3806.
75. Jain, R.K., Dreisbach, J.H., and Spain, J.C., 1994, Biodegradation of *p*-nitrophenol via 1,2,4-benzenetriol by an *Arthrobacter* sp. *Appl. Environ. Microbiol.*, 60:3030–3032.
76. James, K.D., Hughes, M.A., and Williams, P.A., 2000, Cloning and expression of *ntnD*, encoding a novel NAD(P)⁺-independent 4-nitrobenzyl alcohol dehydrogenase from *Pseudomonas* sp. strain TW3. *J. Bacteriol.*, 182:3136–3141.
77. James, K.D. and Williams, P.A., 1998, *ntn* Genes determining the early steps in the divergent catabolism of 4-nitrotoluene and toluene in *Pseudomonas* sp. strain TW3. *J. Bacteriol.*, 180:2043–2049.
78. Jensen, H.L. and Lautrup-Larsen, G., 1967, Microorganisms that decompose nitro-aromatic compounds, with special reference to dinitro-ortho-cresol. *Acta Agr. Scand.*, 17:115–126.
79. Jerger, D.E. and Woodhull, P., 2000, Applications and costs for biological treatment of explosive-contaminated soils in the United States. In J.C. Spain, J.B. Hughes, and H.-J. Knackmuss (eds), *Biodegradation of Nitroaromatic Compounds and Explosives*, pp. 395–423. Lewis Publishers, Boca Raton.
80. Johnson, G.R., Jain, R.K., and Spain, J.C., 2000, Properties of the trihydroxytoluene oxyge-nase from *Burkholderia cepacia* R34: An extradiol dioxygenase from the 2,4-dinitrotoluene pathway. *Arch. Microbiol.*, 173:86–90.
81. Johnson, G.R., Jain, R.K., and Spain, J.C., 2002, Origins of the 2,4-dinitrotoluene pathway. *J. Bacteriol.*, 184:4219–4232.
82. Johnson, G.R. and Spain, J.C., 2003, Evolution of catabolic pathways for synthetic compounds: Bacterial pathways for degradation of 2,4-dinitrotoluene and nitrobenzene. *Appl. Microbiol. Biotechnol.*, 62:110–123.
83. Kadiyala, V., Nadeau, L.J., and Spain, J.C., 2003, Construction of *Escherichia coli* strains for conversion of nitroacetophenones to *ortho*-aminophenols. *Appl. Environ. Microbiol.*, 69:6520–6526.

84. Kadiyala, V., Smets, B.F., Chandran, K., and Spain, J.C., 1998, High affinity *p*-nitrophenol oxidation by *Bacillus sphaericus* JS905. *FEMS Microbiol. Lett.*, 166:115–120.
85. Kadiyala, V. and Spain, J.C., 1998, A two-component monooxygenase catalyzes both the hydroxylation of *p*-nitrophenol and the oxidative release of nitrite from 4-nitrocatechol in *Bacillus sphaericus* JS905. *Appl. Environ. Microbiol.*, 64:2479–2484.
86. Katsivela, E., Wray, V., Pieper, D.H., and Wittich, R.-M., 1999, Initial reactions in the biodegradation of 1-chloro-4-nitrobenzene by a newly isolated bacterium, strain LW1. *Appl. Environ. Microbiol.*, 65:1405–1412.
87. Kieboom, J., Van den Brink, H., Frankena, J., and de Bont, J.A.M., 2001, Production of 3-nitrocatechol by oxygenase-containing bacteria: Optimization of the nitrobenzene biotransformation by *Nocardia* S3. *Appl. Microbiol. Biotechnol.*, 55:290–295.
88. Koder, R.L., Haynes, C.A., Rodgers, M.E., Rodgers, D.W., and Miller, A.-F., 2002, Flavin thermodynamics explain the oxygen insensitivity of enteric nitroreductases. *Biochemistry*, 41:14197–14205.
89. Lan, R. and Reeves, P.R., 1996, Gene transfer is a major factor in bacterial evolution. *Mol. Biol. Evol.*, 13:47–55.
90. Lendenmann, U. and Spain, J.C., 1996, 2-Aminophenol 1,6-dioxygenase: A novel aromatic ring cleavage enzyme purified from *Pseudomonas pseudoalcaligenes* JS45. *J. Bacteriol.*, 178:6227–6232.
91. Lenke, H., Achtnich, C., and Knackmuss, H.-J., 2000, Perspectives of bioelimination of polynitroaromatic compounds. In J.C. Spain, J.B. Hughes, and H.-J. Knackmuss (eds), *Biodegradation of Nitroaromatic Compounds and Explosives*, pp. 91–126. Lewis Publishers, Boca Raton.
92. Lenke, H. and Knackmuss, H.-J., 1992, Initial hydrogenation during catabolism of picric acid by *Rhodococcus erythropolis* HL 24-2. *Appl. Environ. Microbiol.*, 58:2933–2937.
93. Lenke, H. and Knackmuss, H.-J., 1996, Initial hydrogenation and extensive reduction of substituted 2,4-dinitrophenols. *Appl. Environ. Microbiol.*, 62:784–790.
94. Lenke, H., Pieper, D.H., Bruhn, C., and Knackmuss, H.-J., 1992, Degradation of 2,4-dinitrophenol by two *Rhodococcus erythropolis* strains, HL 24-1 and HL 24-2. *Appl. Environ. Microbiol.*, 58:2928–2932.
95. Lessner, D.J., Johnson, G.R., Parales, R.E., Spain, J.C., and Gibson, D.T., 2002, Molecular characterization and substrate specificity of nitrobenzene dioxygenase from *Comamonas* sp. strain JS765. *Appl. Environ. Microbiol.*, 68:634–641.
96. Lessner, D.J., Parales, R., Narayan, S., and Gibson, D.T., 2003, Expression of the nitroarene dioxygenase genes in *Comamonas* sp. strain JS765 and *Acidovorax* sp. strain JS42 is induced by multiple aromatic compounds. *J. Bacteriol.*, 185:3895–3904.
97. Leung, K.T., Campbell, S., Gan, Y., White, D.C., Lee, H., and Trevors, J.T., 1999, The role of the *Sphingomonas* species UG30 pentachlorophenol-4-monooxygenase in *p*-nitrophenol degradation. *FEMS Microbiol. Lett.*, 173:247–253.
98. Lorenz, M.G. and Wackernagel, W., 1990, Natural genetic transformation of *Pseudomonas stutzeri* by sand-adsorbed DNA. *Appl. Microbiol.*, 154:380–385.
99. Mason, R.P. and Josephy, P.D., 1985, Free radical mechanism of nitroreductase. In D.E. Rickert (ed.), *Toxicity of Nitroaromatic Compounds*, pp. 121–140. Hemisphere Publishing Corporation, Washington, D. C.
100. Meulenberg, R. and de Bont, J.A.M., 1995, Microbial production of catechols from nitroaromatic compounds. In J. C. Spain (ed.), *Biodegradation of Nitroaromatic Compounds*, pp. 37–52. Plenum Publishing Corp., New York.
101. Meulenberg, R., Pepi, M., and de Bont, J.A.M., 1996, Degradation of 3-nitrophenol by *Pseudomonas putida* B2 occurs via 1,2,4-benzenetriol. *Biodegradation*, 7:303–311.

102. Michán, C., Delgado, A., Haïdour, A., Lucchesi, G., and Ramos, J.L., 1997, In vivo construction of a hybrid pathway for metabolism of 4-nitrotoluene in *Pseudomonas fluorescens*. *J. Bacteriol.*, 63:3036–3038.
103. Mironov, A.D., Krest'yaninov, V.Y., Korzhenevich, V.I., Evtushenko, I.Y., and Barkovskii, A.L., 1992, Degradation of 2-nitrobenzoic acid and other aromatic compounds by a *Pseudomonas pseudoalcaligenes* strain. *Appl. Biochem. Microbiol.*, 27:433–438.
104. Muraki, T., Taki, M., Hasegawa, Y., Iwaki, H., and Lau, P.C.K., 2003, Prokaryotic homologs of the eukaryotic 3-hydroxyanthranilate 3,4-dioxygenase and 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase in the 2-nitrobenzoate degradation pathway of *Pseudomonas fluorescens* strain KU-7. *Appl. Environ. Microbiol.*, 69:1564–1572.
105. Nadeau, L.J., He, Z., and Spain, J.C., 2000, Production of 2-amino-5-phenoxyphenol from 4-nitrobiphenyl ether using nitrobenzene nitroreductase and hydroxylaminobenzene mutase from *Pseudomonas pseudoalcaligenes* JS45. *J. Ind. Microbiol. Biotechnol.*, 24:301–305.
106. Nadeau, L.J., He, Z., and Spain, J.C., 2003, Bacterial conversion of hydroxylamino aromatic compounds by both lyase and mutase enzymes involves intramolecular transfer of hydroxyl groups. *Appl. Environ. Microbiol.*, 69:2786–2793.
107. Nadeau, L.J. and Spain, J.C., 1995, Bacterial degradation of *m*-nitrobenzoic acid. *Appl. Environ. Microbiol.*, 61:840–843.
108. Nishino, S.F., Paoli, G., and Spain, J.C., 2000, Aerobic degradation of dinitrotoluenes and pathway for bacterial degradation of 2,6-dinitrotoluene. *Appl. Environ. Microbiol.*, 66:2139–2147.
109. Nishino, S.F. and Spain, J.C., 1993, Cell density-dependent adaptation of *Pseudomonas putida* to biodegradation of *p*-nitrophenol. *Environ. Sci. Technol.*, 27:489–494.
110. Nishino, S.F. and Spain, J.C., 1993, Degradation of nitrobenzene by a *Pseudomonas pseudoalcaligenes*. *Appl. Environ. Microbiol.*, 59:2520–2525.
111. Nishino, S.F. and Spain, J.C., 1995, Oxidative pathway for the biodegradation of nitrobenzene by *Comamonas* sp. strain JS765. *Appl. Environ. Microbiol.*, 61:2308–2313.
112. Nishino, S.F., Spain, J.C., Lenke, H., and Knackmuss, H.-J., 1999, Mineralization of 2,4- and 2,6-dinitrotoluene in soil slurries. *Environ. Sci. Technol.*, 33:1060–1064.
113. Padda, R.S., Wang, C.Y., Hughes, J.B., and Bennett, G.N., 2000, Mutagenicity of trinitrotoluene and metabolites formed during anaerobic degradation by *Clostridium acetobutylicum* ATCC 824. *Environ. Toxicol. Chem.*, 19:2871–2875.
114. Pak, J.W., Knoke, K.L., Noguera, D.R., Fox, B.G., and Chambliss, G.H., 2000, Transformation of 2,4,6-trinitrotoluene by purified xenobiotic reductase B from *Pseudomonas fluorescens* I-C. *Appl. Environ. Microbiol.*, 66:4742–4750.
115. Parales, J.V., Kumar, A., Parales, R.E., and Gibson, D.T., 1996, Cloning and sequencing of the genes encoding 2-nitrotoluene dioxygenase from *Pseudomonas* sp. JS42. *Gene*, 181:57–61.
116. Parales, J.V., Parales, R.E., Resnick, S.M., and Gibson, D.T., 1998, Enzyme specificity of 2-nitrotoluene 2,3-dioxygenase from *Pseudomonas* sp. strain JS42 is determined by the C-terminal region of the a subunit of the oxygenase component. *J. Bacteriol.*, 180:1194–1199.
117. Parales, R.E., 2000, Molecular biology of nitroarene degradation. In J.C. Spain, J. B. Hughes, and H.-J. Knackmuss (eds), *Biodegradation of Nitroaromatic Compounds and Explosives*, pp. 63–89. Lewis Publishers, Boca Raton.
118. Parales, R.E., Emig, M.D., Lynch, N.A., and Gibson, D.T., 1998, Substrate specificities of hybrid naphthalene and 2,4-dinitrotoluene dioxygenase enzyme systems. *J. Bacteriol.*, 180:2337–2344.
119. Parales, R.E., Ontl, T.A., and Gibson, D.T., 1997, Cloning and sequence analysis of a catechol 2,3-dioxygenase gene from the nitrobenzene-degrading strain *Comamonas* sp JS765. *J. Ind. Microbiol. Biotechnol.*, 19:385–391.

- 119a. Parales, R.E. and Resnick, S.M. In press. Aromatic hydrocarbon dioxygenases. In: *Soil Biology, vol 2, Biodegradation and Bioremediation*, A.J. Singh and O.P. Ward, (eds.) Springer-Verlag, Germany.
120. Park, H.-S. and Kim, H.-S., 2000, Identification and characterization of the nitrobenzene catabolic plasmids pNB1 and pNB2 in *Pseudomonas putida* HS12. *J. Bacteriol.*, 182:573–580.
121. Park, H.-S. and Kim, H.-S., 2001, Genetic and structural organization of the aminophenol catabolic operon and its implication for evolutionary process. *J. Bacteriol.*, 183:5074–5081.
122. Park, H.-S., Lim, S.-J., Chang, Y.K., Livingston, A.G., and Kim, H.-S., 1999, Degradation of chloronitrobenzenes by a coculture of *Pseudomonas putida* and a *Rhodococcus* sp. *Appl. Environ. Microbiol.*, 65:1083–1091.
123. Peres, C.M., Naveau, H., and Agathos, S.N., 1999, Cross induction of 4-nitrobenzoate and 4-aminobenzoate degradation by *Burkholderia cepacia* strain PB4. In R. Fass, Y. Flashner, and S. Reuveny (eds), *Novel Approaches for Bioremediation of Organic Pollution*, pp. 71–81. Plenum Publishers, New York.
124. Peres, C.M., Russ, R., Lenke, H., and Agathos, S.N., 2001, Biodegradation of 4-nitrobenzoate, 4-aminobenzoate and their mixtures: New strains, unusual metabolites and insights into pathway regulation. *FEMS Microbiol. Ecol.*, 37:151–159.
125. Peres, C.M., Van Aken, B., Naveau, H., and Agathos, S.N., 1999, Continuous degradation of mixtures of 4-nitrobenzoate and 4-aminobenzoate by immobilized cells of *Burkholderia cepacia* strain PB4. *Appl. Microbiol. Biotechnol.*, 52:440–445.
126. Prakash, D., Chauhan, A., and Jain, R.K., 1996, Plasmid-encoded degradation of *p*-nitrophenol by *Pseudomonas cepacia*. *Biochem. Biophys. Res. Comm.*, 224:375–381.
127. Preuss, A., Fimpel, J., and Diekert, G., 1993, Anaerobic transformation of 2,4,6-trinitrotoluene (TNT). *Arch. Microbiol.*, 159:345–353.
128. Preuss, A. and Rieger, P.-G., 1995, Anaerobic transformation of 2,4,6-trinitrotoluene and other nitroaromatic compounds. In J. C. Spain (ed.), *Biodegradation of Nitroaromatic Compounds*, pp. 69–85. Plenum Publishing Corp., New York.
129. Ramanathan, M.P. and Lalithakumari, D., 1999, Complete mineralization of methylparathion by *Pseudomonas* sp. A3. *Appl. Biochem. Biotechnol.*, 80:1–12.
130. Rani, L. and Lalithakumari, D., 1994, Degradation of methyl parathion by *Pseudomonas putida*. *Can. J. Microbiol.*, 40:1000–1006.
131. Rhys-Williams, W., Taylor, S.C., and Williams, P.A., 1993, A novel pathway for the catabolism of 4-nitrotoluene by *Pseudomonas*. *J. Gen. Microbiol.*, 139:1967–1972.
132. Rieger, P.-G. and Knackmuss, H.-J., 1995, Basic knowledge and perspectives on biodegradation of 2,4,6-trinitrotoluene and related nitroaromatic compounds in contaminated soil. In J. C. Spain (ed.), *Biodegradation of Nitroaromatic Compounds*, pp. 1–18. Plenum Publishing Corp., New York.
133. Rieger, P.-G., Sinnwell, V., Preuß, A., Francke, W., and Knackmuss, H.-J., 1999, Hydride-Meisenheimer complex formation and protonation as key reactions of 2,4,6-trinitrophenol biodegradation by *Rhodococcus erythropolis*. *J. Bacteriol.*, 181:1189–1195.
134. Samanta, S.K., Bhushan, B., Chauhan, A., and Jain, R.K., 2000, Chemotaxis of a *Ralstonia* sp. SJ98 toward different nitroaromatic compounds and their degradation. *Biochem. Biophys. Res. Comm.*, 269:117–123.
135. Schackmann, A. and Müller, R., 1991, Reduction of nitroaromatic compounds by different *Pseudomonas* species under aerobic conditions. *Appl. Microbiol. Biotechnol.*, 34:809–813.
136. Schäfer, A., Harms, H., and Zehnder, A.J.B., 1996, Biodegradation of 4-nitroanisole by two *Rhodococcus* spp. *Biodegradation*, 7:249–255.
137. Schenzle, A., Lenke, H., Fischer, P., Williams, P.A., and Knackmuss, H.-J., 1997, Catabolism of 3-nitrophenol by *Ralstonia eutropha* JMP 134. *Appl. Environ. Microbiol.*, 63:1421–1427.

138. Schenzle, A., Lenke, H., Spain, J.C., and Knackmuss, H.-J., 1999, 3-Hydroxylaminophenol mutase from *Ralstonia eutropha* JMP134 catalyzes a Bamberger rearrangement. *J. Bacteriol.*, 181:1444–1450.
139. Schenzle, A., Lenke, H., Spain, J.C., and Knackmuss, H.-J., 1999, Chemoselective nitro group reduction and reductive dechlorination initiate degradation of 2-chloro-5-nitrophenol by *Ralstonia eutropha* JMP134. *Appl. Environ. Microbiol.*, 65:2317–2323.
140. Siciliano, S.D., Gong, P., Sunahara, G.I., and Greer, C.W., 2000, Assessment of 2,4,6-trinitrotoluene toxicity in field soils by pollution-induced community tolerance, denaturing gradient gel electrophoresis, and seed germination assay. *Environ. Toxicol. Chem.*, 19:2154–2160.
141. Somerville, C.C., Nishino, S.F., and Spain, J.C., 1995, Purification and characterization of nitrobenzene nitroreductase from *Pseudomonas pseudoalcaligenes* JS45. *J. Bacteriol.*, 177:3837–3842.
142. Spain, J.C. (ed.), 1995, *Biodegradation of Nitroaromatic Compounds*. Plenum Publishing Corp., New York.
143. Spain, J.C., 1995, Biodegradation of nitroaromatic compounds. *Ann. Rev. Microbiol.*, 49:523–555.
144. Spain, J.C., 1995, Bacterial degradation of nitroaromatic compounds under aerobic conditions. In J. C. Spain (ed.), *Biodegradation of Nitroaromatic Compounds*, pp. 19–35. Plenum Publishing Corp., New York.
145. Spain, J.C. and Gibson, D.T., 1991, Pathway for biodegradation of *p*-nitrophenol in a *Moraxella* sp. *Appl. Environ. Microbiol.*, 57:812–819.
146. Spain, J.C., Hughes, J.B., and Knackmuss, H.-J. (eds), 2000, *Biodegradation of Nitroaromatic Compounds and Explosives*. Lewis Publishers, Boca Raton.
147. Spain, J.C., Wyss, O., and Gibson, D.T., 1979, Enzymatic oxidation of *p*-nitrophenol. *Biochem. Biophys. Res. Comm.*, 88:634–641.
148. Spanggord, R.J., Spain, J.C., Nishino, S.F., and Mortelmans, K.E., 1991, Biodegradation of 2,4-dinitrotoluene by a *Pseudomonas* sp. *Appl. Environ. Microbiol.*, 57:3200–3205.
149. Spiess, T., Desiere, F., Fischer, P., Spain, J.C., Knackmuss, H.-J., and Lenke, H., 1998, A new 4-nitrotoluene degradation pathway in a *Mycobacterium* strain. *Appl. Environ. Microbiol.*, 64:446–452.
150. Suen, W.-C., Haigler, B.E., and Spain, J.C., 1996, 2,4-Dinitrotoluene dioxygenase from *Burkholderia* sp. strain DNT: Similarity to naphthalene dioxygenase. *J. Bacteriol.*, 178:4926–4934.
151. Suen, W.-C. and Spain, J.C., 1993, Cloning and characterization of *Pseudomonas* sp. strain DNT genes for 2,4-dinitrotoluene degradation. *J. Bacteriol.*, 175:1831–1837.
152. Takenaka, S., Murakami, S., Kim, Y.-J., and Aoki, K., 2000, Complete nucleotide sequence and functional analysis of the genes for 2-aminophenol metabolism from *Pseudomonas* sp. AP-3. *Arch. Microbiol.*, 174:265–272.
153. Takenaka, S., Murakami, S., Shinke, R., and Aoki, K., 1998, Metabolism of 2-aminophenol by *Pseudomonas* sp. AP-3: Modified *meta*-cleavage pathway. *Arch. Microbiol.*, 170:132–137.
154. Takenaka, S., Murakami, S., Shinke, R., Hatakeyama, K., Yukawa, H., and Aoki, K., 1997, Novel genes encoding 2-aminophenol 1,6-dioxygenase from *Pseudomonas* species AP-3 growing on 2-aminophenol and catalytic properties of the purified enzyme. *J. Biol. Chem.*, 272:14727–14732.
155. Tewfik, M.S. and Evans, W.C., 1966, The metabolism of 3,5-dinitro-*o*-cresol (DNOC) by soil microorganisms. *Biochem. J.*, 99:31–32.
156. Van Aken, B. and Agathos, S.N., 2001, Biodegradation of nitro-substituted explosives by white-rot fungi: A mechanistic approach. *Adv. Appl. Microbiol.*, 48:1–77.
157. van der Meer, J.R., de Vos, W.M., Harayama, S., and Zehnder, A.J.B., 1992, Molecular mechanisms of genetic adaptation to xenobiotic compounds. *Microbiol. Rev.*, 56:677–694.

158. Vorbeck, C., Lenke, H., Fischer, P., and Knackmuss, H.-J., 1994, Identification of a hydride-Meisenheimer complex as a metabolite of 2,4,6-trinitrotoluene by a *Mycobacterium* strain. *J. Bacteriol.*, 176:932–934.
159. Vorbeck, C., Lenke, H., Fischer, P., Spain, J.C., and Knackmuss, H.-J., 1998, Initial reductive reactions in aerobic microbial metabolism of 2,4,6-trinitrotoluene. *Appl. Environ. Microbiol.*, 64:246–252.
160. Wang, J., Zhou, J.-T., Zhang, J.-S., Zhang, A.-L., and Lu, H., 2001, Aerobic degradation of nitrobenzene by *Pseudomonas* sp. JX165 and its intact cells. *China Environ. Sci.*, 21:144–147.
161. Watrous, M.M., Clark, S., Kutty, R., Huang, S., Rudolph, F.B., Hughes, J.B., and Bennett, G.N., 2003, 2,4,6-Trinitrotoluene reduction by an Fe-only hydrogenase in *Clostridium acetobutylicum*. *Appl. Environ. Microbiol.*, 69:1542–1547.
162. Williams, P.A. and Sayers, J.R., 1994, The evolution of pathways for aromatic hydrocarbon oxidation in *Pseudomonas*. *Biodegradation*, 5:195–217.
163. Won, W.D., Heckly, R.J., Glover, D.J., and Hoffsommer, J.C., 1974, Metabolic disposition of 2,4,6-trinitrotoluene. *Appl. Microbiol.*, 27:513–516.
164. Wright, B.E., 2000, A biochemical mechanism for nonrandom mutations and evolution. *J. Bacteriol.*, 182:2993–3001.
165. Yabannavar, A.V. and Zylstra, G.J., 1995, Cloning and characterization of the genes for *p*-nitrobenzoate degradation from *Pseudomonas pickettii* YH105. *Appl. Environ. Microbiol.*, 61:4284–4290.
166. Zablotowicz, R.M., Leung, K.T., Alber, T., Cassidy, M.B., Trevors, J.T., Lee, H., Veldhuis, L., and Hall, J.C., 1999, Degradation of 2,4-dinitrophenol and selected nitroaromatic compounds by *Sphingomonas* sp. UG30. *Can. J. Microbiol.*, 45:840–848.
167. Zeyer, J. and Kearney, P.C., 1984, Degradation of *o*-nitrophenol and *m*-nitrophenol by a *Pseudomonas putida*. *J. Agric. Food Chem.*, 32:238–242.
168. Zeyer, J. and Kocher, H.P., 1988, Purification and characterization of a bacterial nitrophenol oxygenase which converts *ortho*-nitrophenol to catechol and nitrite. *J. Bacteriol.*, 170:1789–1794.
169. Zeyer, J., Kocher, H.P., and Timmis, K.N., 1986, Influence of *para*-substituents on the oxidative metabolism of *o*-nitrophenols by *Pseudomonas putida* B2. *Appl. Environ. Microbiol.*, 52:334–339.
170. Zhao, J.-S., Singh, A., Huang, X.-D., and Ward, O.P., 2000, Biotransformation of hydroxyl-aminobenzene and aminophenol by *Pseudomonas putida* 2NP8 cells grown in the presence of 3-nitrophenol. *Appl. Environ. Microbiol.*, 66:2336–2342.
171. Zhao, J.-S. and Ward, O.P., 2001, Substrate selectivity of a 3-nitrophenol-induced metabolic system in *Pseudomonas putida* 2NP8 transforming nitroaromatic compounds into ammonia under aerobic conditions. *Appl. Environ. Microbiol.*, 67:1388–1115.
172. Zhou, N.-Y., Al-Dulayymi, J., Baird, M.S., and Williams, P.A., 2002, Salicylate 5-hydroxylase from *Ralstonia* sp. strain U2: A monooxygenase with close relationships to and shared electron transport proteins with naphthalene dioxygenase. *J. Bacteriol.*, 184:1547–1555.
173. Zylstra, G.J., Bang, S.-W., Newman, L.M., and Perry, L., 2000, Microbial degradation of mononitrophenols and mononitrobenzoates. In J.C. Spain, J.B. Hughes, and H.-J. Knackmuss (eds), *Biodegradation of Nitroaromatic Compounds and Explosives*, pp. 145–160. Lewis Publishers, Boca Raton.

SOLVENT-TOLERANCE OF PSEUDOMONADS: A NEW DEGREE OF FREEDOM IN BIOCATALYSIS

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1. INTRODUCTION

Traditional biotechnology can be defined as the use of cellular and molecular processes in microorganisms to manufacture products. Biotechnology is as old as the hills and includes fermentation of beer and wine and production of cheese and bread.

Modern biotechnology is different in a sense that it makes use of the rapidly increasing knowledge of cellular and molecular biology. This knowledge increasingly contributes to solving complex problems and to steering processes towards well-defined end goals. In recent history some important breakthroughs have provided tools for modern biotechnology: (a) discovery of DNA restriction and ligation enzymes, opening the way for gene cloning (1970), (b) establishment of the DNA sequencing technique (1976) and (c) determination of the first full genomic DNA sequence of a unicellular organism, the bacterium *Haemophilus influenzae*, in 1995¹⁴.

The latter event was the starting point for a revolution in biosciences referred to as the genomics era. At a rapidly increasing pace the sequencing and annotation of whole genomes, including the human genome, provides

researchers the tools to study cellular responses and their underlying molecular processes. The genomics era has boosted both investments and revenues in biotechnology industry. Since 1995 the revenues in the United States more than doubled to US\$ 28 billion/year (Ernst & Young LLP, Annual Biotechnology Industry Reports, 1993–2002) and the number of granted patents in the area of biotechnology has tripled to 14,000/year (U.S. Patent and Trademark Office). By far the largest share of these numbers can be contributed to the drug and, to a lesser extent, the food industry. From 1982 to 1994 the number of Food and Drug Administration (FDA) approved biotech drugs in the United States averaged four per year. However, since 1995 this number increased 5 fold to 22 per year.

Modern biotechnology, contrary to the drug industry, still plays a minor role in industrial manufacturing. The production of chemicals by microorganisms or enzymes, referred to as biocatalysis, on a commercial scale is very limited compared to traditional chemical production. It has long been recognized that microorganisms and enzymes have several characteristics which could potentially be favourable for the manufacturing industry. They operate at moderate temperatures, which could reduce energy requirements for a given process. They are highly specific for a given product and thus produce less (toxic) by-products and waste. They may use less purified substrates and do not require expensive chemical activating or protecting groups nor large amounts of solvents.

However, the main obstacle for biocatalysis has been the long time path to come to an efficient and economic process. A process based on biocatalysis can be divided into several distinct phases and disciplines each of which should be passed through (Figure 1).

For this reason most bio-based processes require a time-to-market of several years. Nonetheless, several recent developments have fuelled the expectation that biocatalysis will play an important role in the manufacturing industry in the years to come. First, the advent of genomics technologies has increased, and will do so more in the future, our understanding of molecular processes within the cell. Leads to bottlenecks hampering a given biotransformation will be obtained more readily and solutions to lift these impairments will be generated at a much higher pace than in the pre-genomics era. Second, many bioscientific areas such as genetics, microbiology, biochemistry and process engineering tend to be no longer isolated disciplines but are more integrated, which also shortens the process cycle. The third and decisive development is the increasing attention of the public opinion and governments for industrially sustainable processes⁵³. Reduction of waste and lower consumption of energy and non-renewable materials will have increasing attention in the years to come.

At present, several biocatalytic reactions are performed on an industrial scale by isolated enzymes. Carbohydrases, proteases/peptidases, lipases,

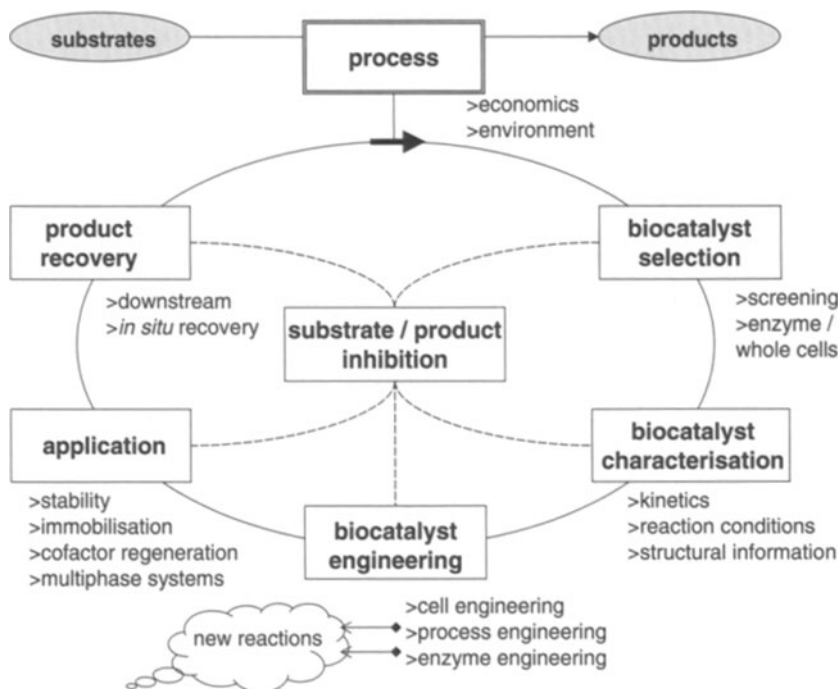


Figure 1. The development of a biocatalytic process: the biocatalysis cycle²⁷.

amidases and lyases make up the majority of enzymes and they are mainly employed by the food/feed, detergent and paper industry.

Whole-cell biocatalysis is also employed but it still has to stand up to its inherent potential. It is evident that biotransformations by whole-cells will be particularly useful in those areas where isolated enzymes are less appropriate. Reactions that require multicomponent enzyme systems and/or cofactor-dependent enzymatic reactions, such as oxidations and reductions, would be preferably performed by whole cells to allow for cheap cofactor regeneration. Chemical compounds such as hydroxylated aromatics, aldehydes, alcohols and epoxides are examples of products that could be synthesized by such reactions. They are important ingredients for (fine)-chemical materials and serve a huge market. Currently, the vast majority of such compounds is produced from fossil resources by production methods that often involve expensive chemical activating/protecting groups and/or large amounts of solvents. Whole-cell biocatalysis could prove an excellent 'green' alternative either transforming one intermediate compound to the desired product in one or few steps or even produce them from renewable natural sources, for example, sugars.

2. TOXICITY OF ORGANIC SOLVENTS AND SOLVENT-TOLERANT PSEUDOMONADS

A major obstacle for whole-cell production of these chemical compounds, also referred to as organic solvents, is their extreme toxicity to living cells. Many organic solvents are quite hydrophobic compounds that exert their toxicity by accumulating in the bacterial cell-membrane⁹⁹. The cell-membrane performs vital functions. It is a matrix for proteins and a selective barrier for solutes between the cell and its environment. Additionally bacterial cytoplasmic membranes serve as a source of energy for the cell by generating a gradient of ions such as protons hereby maintaining a ΔpH and electric potential $\Delta\psi$ which results in the electron motive force.

If a solvent in the membrane reaches a critical concentration then the fluidity of the membrane is disturbed to such an extent that the lipid order and lipid bilayer is destabilized¹⁰⁶. This causes destruction of all structural and functional properties, including energy supply and membrane-embedded transport systems. The devastating effects of solvents on bacterial membranes was already shown in the early 1970s. The presence of toluene was found to impair growth of *Escherichia coli* cells and caused leakage of RNA, phospholipids and proteins^{39, 112}. The disintegration of cell membranes after exposure to an organic solvent was also made visible by de Smet *et al.* in 1978¹⁰⁰. Electron microscopic pictures of toluene treated *E. coli* cells showed considerable damage to the inner membrane.

Hydrophobicity of solvents is usually expressed in terms of $\log\text{Po/w}$, in which \log is the common logarithm and Po/w stands for the partitioning of a given compound over an octanol/water two-phase system. Organic solvents with a $\log\text{Po/w}$ between 1 and 4 are generally regarded as very toxic to living cells^{76, 98}. Solvents such as toluene ($\log\text{P} = 2.5$) or benzene ($\log\text{P} = 2.0$) will readily accumulate into the cell membrane above lethal concentrations. A relationship between $\log\text{Po/w}$ and the logarithm of partitioning of a solvent over a membrane/water two-phase system was established by Sikkema *et al.*, 1994⁹⁸:

$$\text{LogP}_{\text{membr./buffer}} = 0.97 \times \text{LogPo/w} - 0.64 \quad (\text{For solvents with a } \log\text{Po/w} \text{ between 1 and 4.5})$$

From this equation the accumulation of a given solvent into a bacterial membrane can be estimated (Table 1). In a watery system with saturated amounts of toluene (6.3 mM) the maximum amount solved in a bacterial membrane would be 368 mM. A 6.3 mM solution of benzene in water/medium would result in a membrane concentration of 126 mM. In this sense benzene is less toxic than toluene. However, as the solubility of benzene in water is four times higher than the solubility of toluene; saturated amounts of benzene are more toxic to living cells.

Table 1. Accumulation of different solvents in the membrane at saturating concentrations in water.

Solvent	Maximum concentration (mM)		
	LogPo/w	In water	In membrane ^a
Butanol	0.8	1000	1100
Benzene	2.13	23	620
1-Octanol	2.81	4.2	529
Toluene	2.48	6.3	368
Decanol	4.00	0.25	434
Styrene	2.89	3	280

^aAs calculated from: $\text{LogP}_{\text{membr./buffer}} = 0.97 \times \text{LogPo/w} - 0.64$ (Sikkema *et al.*, 1994).

Table 2. Overview of organic solvent-tolerant *Pseudomonas* strains.

Strain	Solvent tolerated ^a	LogPo/w ^b	References
<i>P. putida</i> IH-2000	Toluene	2.5	[35]
<i>P. putida</i> S12	Toluene	2.5	[107]
<i>P. putida</i> DOT-T1	Toluene	2.5	[85]
<i>P. putida</i> Idaho	Toluene	2.5	[7]
<i>P. putida</i> GM73	Toluene	2.5	[46]
<i>P. putida</i> KT2442	<i>p</i> -Xylene	3.1	[15]
<i>P. putida</i> F1	<i>p</i> -Xylene	3.1	[75]
<i>P. putida</i> CE2010	Toluene	2.5	[75]
<i>P. putida</i> PpG1-7T	Toluene	2.5	[96]
<i>P. putida</i> No.69-3	Heptanol	2.4	[103]
<i>P. aeruginosa</i> PST-01	Cyclohexane	3.2	[74]
<i>P. aeruginosa</i> PAO1	Hexane	3.5	[55]
<i>P. aeruginosa</i> LST-03	Toluene	2.5	[73]
<i>P. aeruginosa</i> PAK101	Hexane	3.5	[50]
<i>P. aeruginosa</i> PAK102	<i>p</i> -Xylene	3.1	[50]
<i>P. mendocina</i> K08-1	Dimethylphthalate	2.3	[32]
<i>P. mendocina</i> LF-1	Dimethylphthalate	2.3	[32]
<i>Pseudomonas</i> sp. LB400	Cyclohexane	3.2	[75]

^aTolerance is presented as the ability to grow in the presence of supersaturating amounts of the solvent.

^bLogPo/w values as published by Laane *et al.*⁵²

It was for their very effective antimicrobial properties that solvents like toluene have long been employed as a disinfectant until their severe toxicity for humans was established. The isolation of a solvent-tolerant *Pseudomonas putida* strain by Inoue and Horikoshi in 1989³⁵ that was able to thrive in aqueous medium in the presence of supersaturated amounts of toluene was quite a blow to the perception at that time that such severe stress conditions were not consistent with life. Shortly following this finding other groups also reported the isolation of natural solvent-tolerant bacteria of which the majority belonged to the genus *Pseudomonas* (Table 2), although other gram-negatives and even some gram-positives have also been found to be solvent-tolerant^{65, 66, 75, 77, 61}.

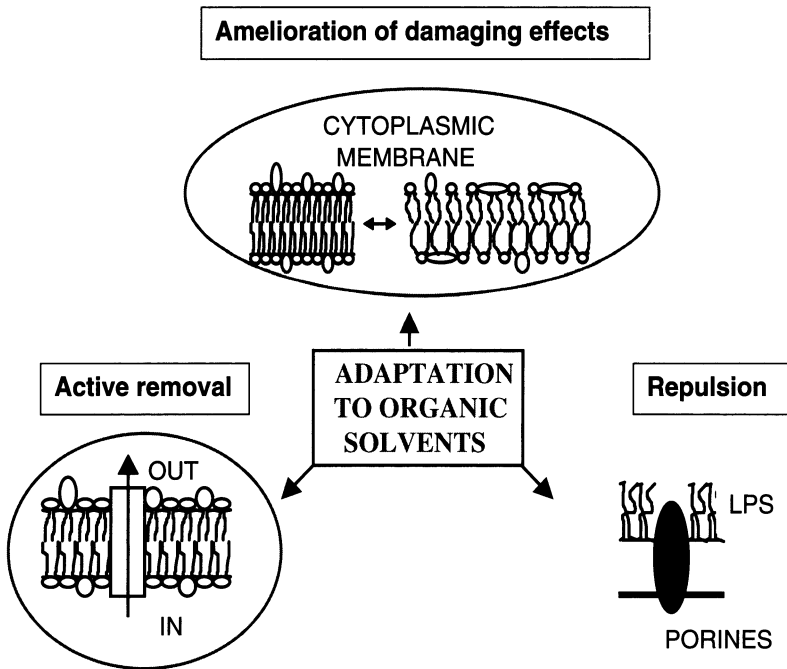


Figure 2. Principal defense strategies of solvent-tolerant bacteria to combat solvent toxicity.

3. MECHANISMS OF SOLVENT-TOLERANCE: PHYSIOLOGIC ADAPTATION

Solvent-tolerant *Pseudomonas* strains have been shown to engage specific physiologic responses to cope with devastating effects of toxic solvents. The details of many of these adaptation mechanisms have been extensively reviewed^{21, 37, 43, 86, 87, 106} and an overview will be given below.

Not surprisingly adaptational responses mainly deal with membrane (both cytoplasmic and outer membrane) associated phenomena. Basically *Pseudomonas* employs three lines of defense to survive solvent stress: (a) repulsion of solvent molecules from the cell, (b) amelioration of damaging effects of solvents and (c) active removal of the solvent from the cell (Figure 2).

3.1. Repulsion of Solvent Molecules from the Cell

Repulsion of solvents from the cell could be considered as the first mechanism in a row of adaptational responses. It is mainly brought about by mechanisms confined to the outer membrane of gram-negatives. The outer membrane

has been shown an effective barrier for hydrophobic molecules⁷² and this is presumed to be the reason why gram-negatives are better able to deal with organic solvent stress than gram-positives. Although the outer membrane can be considered as a highly porous layer carrying many different protein channels (porins) that facilitate diffusion of molecules, its high degree of impermeability to organic solvents is based on its relatively hydrophilic character. This is mainly brought about by the presence of lipopolysaccharides (LPS) which have a low permeability for hydrophobic compounds^{23, 72, 104, 115}. It was observed that *P. putida*, if challenged with solvents, further increase the impermeability of the outer cell membrane by changing the composition of LPS^{1, 47, 81}.

The presence or absence of proteins in the outer-membrane also greatly affects solvent-tolerance. It was shown that loss of porins, such as OprL from *P. putida* DOT-T1 and OprF from *P. aeruginosa* inflicted solvent sensitivity^{54, 89, 90}. As these porins are not only functional as channels but are also considered to stabilize the membrane structure¹¹³ it could be hypothesized that this solvent sensitivity was a result of impairment of the membrane structure. In addition, it was argued that loss of proteins would add to an increase of the cell surface hydrophobicity.

Despite the outer membrane being an effective barrier to solvents, extensive challenging of cells with high enough doses will eventually lead to accumulation of lethal amounts in the cytoplasmic membrane. Rather than being an absolute barrier the outer membrane could be regarded as a means to delay leakage of solvents into the cell, allowing time to engage subsequent lines of defense.

3.2. Amelioration of Damaging Effects of Solvents

Amelioration of the damaging effects is based on counteracting the changes in fluidity of the cytoplasmic membrane inflicted by the solvent. This regulation of fluidity, also referred to as homeoviscous adaptation⁹⁷, was shown to be brought about by several mechanisms:

3.2.1. Change of the Saturation Degree of Membrane Fatty Acids

In bacteria that are not qualified as solvent-tolerant, such as *E. coli*, it was shown that exposure to polar solvents like alcohol and acetone caused an increase of the ratio of saturated/unsaturated fatty acids in the membrane^{33, 34}. Exposure to apolar solvents like benzene had the opposite effect. This effect was also observed in solvent-tolerant *P. putida* upon exposure to toluene and it was shown to be part of the adaptational response^{81, 108}.

3.2.2. Cis/Trans Isomerization of Unsaturated Fatty Acids

Isomerization of the *cis* double bond of unsaturated membrane fatty acids into the *trans* configuration was observed in *P. putida* challenged with

organic solvents^{6, 42}. This isomerization brings about a higher ordering of the membrane structure hereby decreasing its fluidity. It was shown that the extent of isomerization depended on the toxicity and the concentration of the organic solvent used. *Cis-trans* isomerization was shown to be cofactor independent and was not related with lipid biosynthesis^{11, 19, 20, 24, 64}. The gene encoding for *cis-trans* isomerase (*cti*) from *P. putida* was first isolated and sequenced by Holtwick *et al.*²⁵. After this finding several other *cti*-like genes have been identified mainly from *Pseudomonas* strains⁴¹.

3.2.3. Changing Phospholipids Head Groups

Another mechanism that involves regulation of cytoplasmic membrane fluidity is based on alteration of composition of the phospholipids head groups^{80, 86, 106}. It was shown that in solvent-tolerant *P. putida* strains the relative amount of diphosphatidylglycerol^{86, 106} or phosphatidylethanolamine⁸⁰ increases upon adaptation to solvents. It was hypothesized that alteration of head group composition affects the phase preference of the lipids. This in turn compensates for the effects of solvents on the fluidity and ordering of the membrane lipids⁸⁶.

3.3. Active Removal of Solvents from the Cell

The idea that solvents are actively extruded from the cytoplasmic membrane of solvent-tolerant bacteria originated from the notion that modifications in the outer and inner membrane solely would not prevent the eventual accumulation of solvents in the cell to lethal levels. Indeed in 1996 it was established for the first time that toluene was actively removed from the solvent-tolerant *P. putida* S12³⁶ in an energy driven (proton motive force) fashion. The genes encoding this efflux system were identified and characterized in 1998⁴⁵. Since then several other solvent efflux systems have been identified and characterized in solvent-tolerant *Pseudomonas* strains^{15, 67, 84, 91}.

Solvent efflux pumps appear to be the principal defence mechanism for extremely toxic solvents like toluene. They were shown to belong to the RND-type (resistance nodulation division) efflux pumps that till recently were especially known to be involved in antibiotic resistance in e.g. *P. aeruginosa* (refs. [71, 82] for recent reviews). Indeed several of the efflux systems involved in resistance to antibiotics were also shown to be involved in solvent-tolerance⁵⁵ and vice versa^{15, 84, 91}.

Based on studies of the structure of the AcrAB-TolC multidrug efflux system of *E. coli*^{51, 69} it is proposed that the structurally related solvent efflux systems consist of a tripartite protein complex that spans the inner and outer membrane. Generally the largest of the 3 proteins is located in the cytoplasmic membrane, where it is presumed to take accumulated solvent molecules.

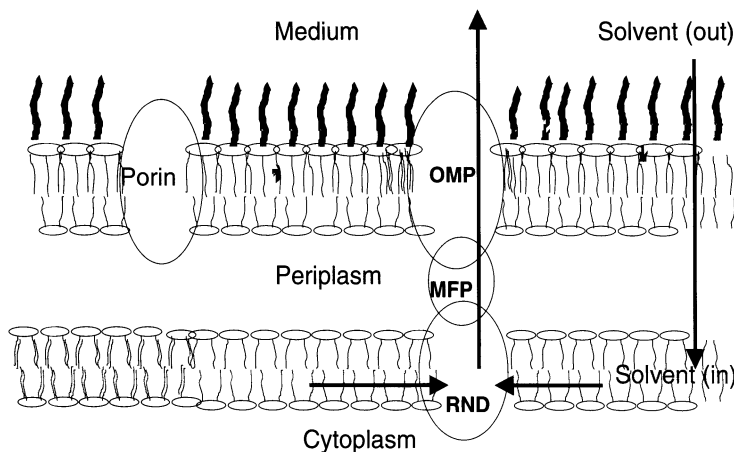


Figure 3. Schematic drawing of a typical solvent efflux system in the membrane of gram-negative bacteria. Arrows indicate extrusion of solvents from inner leaflet of the inner membrane. Abbreviations: MFP, membrane fusion protein; RND, inner membrane transporter protein; OMP, outer membrane protein (porin).

A membrane fusion protein connects the cytoplasmic protein with a porin in the outer membrane facilitating extrusion of the solvent out of the cell into the environment (Figure 3). Although the exact mechanism of solvent extrusion by these proteins remains to be elucidated, the studies on multidrug transporters of the RND type such as AcrAB-TolC and the Mex efflux systems (ref. [71, 82] for recent reviews) may well stand model.

Several studies have indicated that solvent efflux pumps have a broad substrate specificity. It was shown for the *srpABC* pump from *P. putida* S12 that it is involved in the tolerance to aromatic compounds, alcohols, alkanes and cyclo-alkanes, but not to antibiotics³⁸. Recently it was shown that in *P. putida* S12 a different efflux pump, ArpABC, is involved in the active efflux of multiple antibiotics⁴⁴. The broad range solvent-tolerant phenotype of *P. putida* DOT-T1E was shown to be the result of in part overlapping substrate specificity of three identified efflux pumps⁹¹.

Genes encoding efflux systems of the RND type are located adjacent to one or two putative regulatory genes¹⁷. In general these genes encode repressors.

The genetic organization of solvent efflux systems reveals a similar structure and in few instances light was shed on the role of the regulators. The *srpABC* genes in *P. putida* S12 are preceded by two clustered genes *srpS* and *srpR* that by sequence comparison resemble repressor genes (Figure 4). *SrpS* is an IclR type repressor and *srpR* resembles TetR like regulators such as *acrR* (GenBank AF061937), which is a repressor of *acrAB* transcription in *E. coli*⁵⁶. It was shown recently that interruption of the *srpSR* cluster in *P. putida* S12

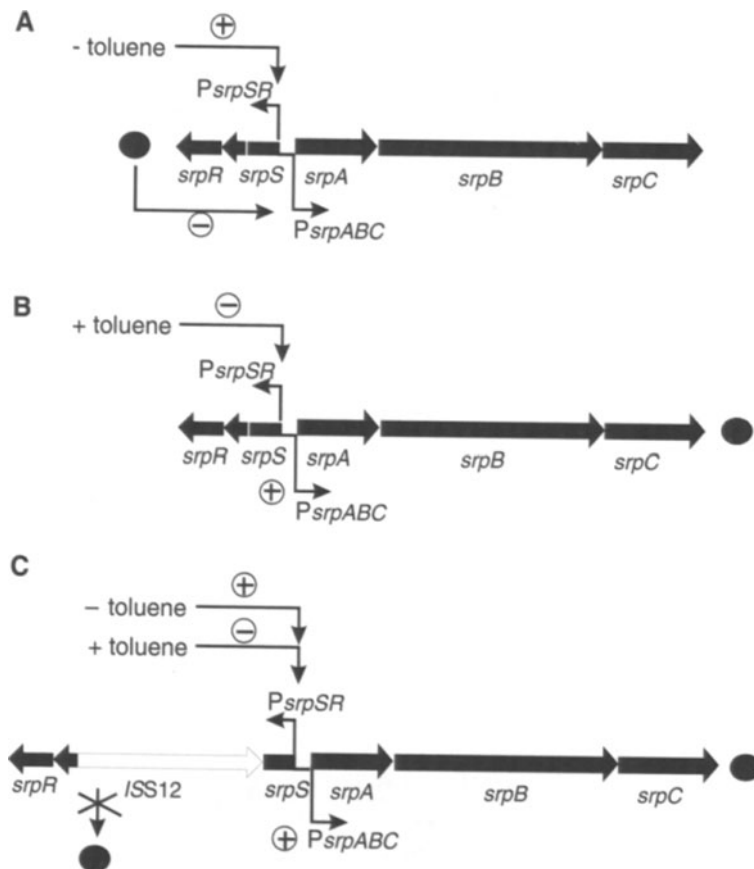


Figure 4. Schematic representation of the genetic organization of the *srp* efflux system in *P. putida* S12, including the regulatory genes. From the work by Wery *et al.* 2001¹⁰⁹ the following picture emerges. (A) The gene products of *srpSR* repress expression of the *srpABC* operon under normal conditions. (B) Exposure to sublethal concentrations of toluene (3mM) lifts repression by SrpSR, yielding toluene adapted cells. (C) In genetic variants of *P. putida* S12 that are solvent shock-tolerant the insertion of *ISS12* in *srpS* structurally impedes *srpSR* expression yielding permanently solvent-adapted cells.

brought about enhanced tolerance to toluene shocks without pre-adaptation to the solvent¹⁰⁹. This tolerance, which was comparable to levels observed in pre-adapted wild-type cells, could be completely reversed after complementation with intact *srpSR*. The results clearly indicated that the gene products of *srpSR* repress expression from *srpABC* under normal conditions.

In *P. putida* DOT-T1E the solvent efflux genes *tigGHI* are preceded by the putative regulatory genes *tigVW* in a manner highly similar to *srpABC/srpSR*. Here the role of the regulators is not yet established in physiological

tests⁹¹. Contrary to *srpABC* and *ttgGHI* the genes encoding the two other efflux systems in DOT-T1E, *ttgABC* and *ttgDEF*, are preceded by only one putative regulatory gene. It was established that the expression from *ttgABC* was negatively controlled by the *ttgR* gene product¹³.

Recently a possible alternative mechanism for active removal of solvents has been described for *P. putida*. It was shown that the solvent-tolerant *P. putida* IH-2000 releases membrane vesicles upon challenging with toluene, whereas a solvent-sensitive mutant did not. As these vesicles were demonstrated to contain higher concentrations of toluene molecules than the cell membrane, they were presumed to be effective scavengers of toluene molecules⁴⁸. This mechanism of solvent removal is difficult to rationalize on the basis of the generally hold idea of membranes presenting a two-dimensional 'fluid mosaic'. In this state, the packing supposedly is loose and lateral diffusion of lipids and proteins and also of solvents is expected to be relatively rapid. However, in recent years more and more information is emerging about the existence of other lipid bilayer states, referred to as 'liquid-ordered' or as also referred to as rafts. These rafts have been studied mainly in eukaryotic systems¹⁶, but also in prokaryotes they may be relevant.

4. MECHANISMS OF SOLVENT-TOLERANCE: GENETIC ADAPTATION

From the above it becomes clear that *Pseudomonas* has evolved many cell functions that enable it to cope with solvent stress and probably more mechanisms will be discovered in the near future aided by the vast amount of information that is generated by the different *Pseudomonas* genome sequencing projects^{70, 101}.

Most of the functions related to solvent-tolerance have evolved during a long period of time and are the result of constant selective pressure imposed by the different habitats of *Pseudomonas*. It was only acknowledged recently that evolution of traits is not necessarily a long-term process, but can be a matter of a limited number of generations, which for bacteria stands for hours rather than ages. Till date little attention has been paid to the phenomenon of accelerated evolution with respect to the emergence of *Pseudomonas* variants that are better prepared for solvent-stress.

The emergence of *Pseudomonas* variants from a genetically homogeneous population that was better prepared to cope with an instant solvent shock was observed in the past¹⁰⁷. Normally, adaptational responses of solvent-tolerant *Pseudomonas* occur after exposure to non-lethal inducing amounts of, for example, toluene (pre-adaptation). In this way, all cells in the population are able to engage their defensive mechanisms and are prepared to

survive even a separate phase of the solvent (1% [vol./vol.]). This solvent-tolerant phenotype is rapidly lost when the population is subsequently incubated in the absence of toluene. If toluene (1% [vol./vol.]) is added shock-wise to cells that are not pre-adapted, then lysis of cells occurs.

Surprisingly, a few cells in the population were consistently found to survive such a shock^{7, 26, 46, 107}. These surviving individuals eventually grew to a high density in the presence of a separate phase of the solvent. Contrary to pre-adapted cells, such population maintained its toluene-tolerant phenotype after prolonged incubation without toluene¹⁰⁷. No adequate explanation could be given for this phenomenon, however, recently it was demonstrated that in *P. putida* S12 an insertion sequence, *ISS12* was involved in the emerging of this toluene-shock tolerant variant¹⁰⁹.

It was found that the variant carried an extra copy of *ISS12* in the putative regulatory gene cluster *srpSR* of the solvent efflux pump *SrpABC* (Figure 4) and evidence was provided that the insertion brought about an increased basal (under normal growth conditions) expression of the solvent pump. This mechanism was shown to be the basis of the high survival frequency of this genetic variant.

Based on these findings it was hypothesized that the *ISS12* was employed by its host as an effective mutator element: it transposes at a certain rate into new genomic locations, hereby sometimes effectuating new phenotypes that, if beneficial under given conditions, result in the rising of a new strain. Thus the bacterium always maintains sub-populations of pre-conditioned cells. Indeed it was found that challenging *P. putida* S12 with other solvents and even antibiotics led to variants with extra *ISS12* copies inserted in different yet to be characterized chromosomal locations (Wery *et al.* unpublished results).

Shortly after this finding Kobayashi *et al.*⁴⁹ found a correlation between enhanced solvent-tolerance and insertion sequences in *E. coli*. In this report a solvent-sensitive mutant was studied that carries a truncated *acrB* gene coding for a part of the *AcrAB-TolC* efflux pump. It was shown that two variants of this mutant with increased solvent-tolerance carried either insertion element *IS1* and *IS30* upstream of the *acrEF* operon.

The *acrEF* genes are normally dormant in *E. coli* and unlike *AcrAB-TolC* do not contribute to increased solvent-tolerance under solvent stress^{57, 58}. It was shown that both variants carried increased amounts of *AcrE* protein, probably as a result of creation of promoters by the insertion elements⁴⁹.

5. *PSEUDOMONAS PUTIDA* IN WHOLE-CELL BIOCATALYSIS

As outlined in Section 2 of this chapter toxicity of products is often a major obstacle for whole-cell biocatalysis. Bacteria, yeasts and fungi, such as

E. coli, *Saccharomyces cerevisiae* and *Aspergillus* sp., which are the most widely used in biocatalytic processes are not necessarily the most appropriate to combat product toxicity. The solvent-tolerant properties of many *Pseudomonas* strains may well be the key in tackling this major problem in several biocatalytic applications.

Pseudomonads already have been shown to possess excellent biocatalytic abilities that are mainly effectuated by a variety of oxygenases which are able to transform thousands of natural and industrial compounds¹⁰⁵. These biocatalytic properties, together with their tolerance to many aromatics, such as benzene, toluene, ethylbenzene and xylenes (BTEX), already were demonstrated to be a strong combination for bioremediation^{9, 83, 85, 92, 105}. However, there are only a limited number of reports describing bioproduction of (fine) chemicals by *Pseudomonas* (Table 3), and only few concepts for a commercial production process for the class of chemicals of interest ($1 < \log P < 4$) are available¹⁰⁵.

This could in part be explained by the fact that most of the wild-type strains described so far do not accumulate industrially useful compounds. If (genetically) modified to perform a given conversion, *Pseudomonas* hosts may, as a result of their broad biocatalytic abilities, degrade a desired product (especially aromatics). Until recently relatively few genetic tools were available for *Pseudomonas* to tackle these problems.

In several instances 'classic' chemical or UV-mutagenesis methods were used to obtain *Pseudomonas* hosts that accumulated a compound of interest. The production of catechols from toluene was studied in *P. putida* 2313. This strain is able to completely mineralize toluene and it was mutagenized with N-methyl-N-nitro-N-nitrosoguanidine (NTG) to obtain a mutant that was blocked in the transformation of 3-methylcatechol in the toluene degradation route. Based on this mutant a fed-batch 3-methylcatechol production system was designed⁸⁸. Similarly, bioconversion systems to transform naphthalene into naphthalene-*cis*-glycol¹⁸ and toluene into toluene-*cis*-glycol⁵ were based on the use of a *P. putida* mutant that was obtained after UV mutagenesis¹⁰².

In several studies the genes for a bioconversion of interest were transferred from *Pseudomonas* to *E. coli*. This host is in many instances not capable of degrading the product; it is more accessible for genetic tools and is much better studied in large scale fermentations^{78, 114}. The preference for *E. coli* as a host in the bio-production of the expensive fine-chemical styrene oxide from the inexpensive bulk-chemical styrene illustrates this phenomenon. Here *E. coli* is used as a host for the expression of the *xylMA*^{79, 114} or the *styAB*⁷⁸ genes from *P. putida* mt-2 and *Pseudomonas* species VLB120, respectively.

However, the use of *P. putida* as a host for these types of bioconversions appears to be attractive and increasingly attainable. The tools for genetic modification such as gene cloning, vector transfer, gene inactivation, mutation and

Table 3. Examples of *Pseudomonas* based bioprocesses with toxic substrates and/or products.

Biocatalyst	Process	LogPo/w ^a	System	[Product](g/L) ^b	References
<i>P. oleovorans</i>	Conversion of 1,7-octadiene to 7,8-epoxy-1-octene and 1,2-7,8-diepoxyoctane	Substrate: 4 Products: 2.7 and 1.4	Single aqueous	1.4	[95]
			Two-liquid water-cyclohexane	7.46	
<i>P. putida</i> 2313	Conversion of benzene to catechol	Substrate: 2.0 Product: 1.0	Single aqueous	3	[88]
<i>P. putida</i> 2313	Conversion of toluene to 3-methylcatechol	Substrate: 2.5 Product: 1.5	Single aqueous	1.3	[88]
<i>P. putida</i> DS10	Conversion of toluene to 3-methylcatechol	Substrate: 2.5 Product: 1.5	Solid adsorption	5	[110]
			Single aqueous	0.9	
			Two-liquid water-octanol	3.1	
<i>P. putida</i> MC2	Conversion of toluene to 3-methylcatechol	Substrate: 2.5 Product: 1.5	Single aqueous	1.74	[28], [29], [30], [31]
			Two-liquid water-octanol	10.3	
			Two-liquid water-octanol, membrane separated	6.9	

<i>P. putida</i> UV4	Conversion of toluene to toluene <i>cis</i> -glycol	Substrate: 2.54 Product: 0.6	Two-liquid water-tetradecane	25 ^c	[5]
<i>P. putida</i> SMA	Conversion of styrene to styrene oxide	Substrate: 2.89 Product: 1.6	Two-liquid water-AL240 ^d	1.9	[79]
<i>P. putida</i> EM2878	Conversion of toluene to <i>p</i> -hydroxybenzoic acid	Substrate: 2.54 Product: 1.4	Single aqueous	0.13	[62]
<i>P. putida</i> GS1	Conversion of limonene to perillic acid	Substrate: 4.8 Product: 3.6	Single aqueous	11	[59]

^aAs calculated according to Laane *et al.*, 1986⁵². Compounds are considered toxic if $1 < \log P_o/w < 4$.

^bIn two-liquid water-solvent systems maximum concentration in the solvent phase is depicted.

^cConcentration in aqueous phase.

^dMixture of alkanes >13 C-atoms.

expression are being developed at a high pace^{10, 30, 79} (ref. [93] for an overview) and the availability of genetic tools no longer pose a limitation for the use of *P. putida* as an efficient biocatalyst. In several studies this was already acknowledged. For the production of styrene oxide from styrene, Panke *et al.*⁷⁹ employed a genetic system based on the expression of xylene monooxygenase by the alkane inducible *alkS/alkBp* promoter in *P. putida* KT2440. This expression cassette was stably integrated via a modified mini-Tn5 transposon in the chromosome of the host to prevent segregational instability. Similarly Wery *et al.*¹¹⁰ reported a concept for the production of 3-methylcatechol from toluene in the solvent-tolerant *P. putida* S12. Genes responsible for this conversion, *todC1C2BAD*, were isolated from *P. putida* F1 that completely mineralizes toluene via 3-methylcatechol^{116, 117}. The genes were placed under control of a salicylate inducible promoter. This expression cassette was conjugated into *P. putida* S12 via the mini-Tn5 transposon system and screening of several mutants yielded a stable 3-methylcatechol producer strain. This concept was further improved by Husken *et al.*^{28–31} who combined the use of this integrative expression system with a mutant derivative of *P. putida* F1 that was no longer able to further degrade 3-methylcatechol.

5.1. Two-Liquid Water-Solvent Production Systems

In processes such as those outlined above it is often imperative to scavenge toxic product away from the biocatalysts. This could be achieved by solid adsorption materials^{22, 88}, but for many processes application of a solvent phase in a two-liquid water-solvent system is to be preferred. In an effective two-liquid water-solvent system the solvent phase acts as a scavenger of a toxic product and as a reservoir for toxic substrates hereby diminishing their toxicity. Moreover, such system leads to concentration of the product and preferably leaves enough substrate in the aqueous liquid phase to bring about sufficient availability. In many cases the use of solvent-sensitive hosts poses limitations upon the choice of the solvent phase of such two-liquid water-solvent systems due to toxicity.

A possible solution to this problem could be separation of the solvent phase from the aqueous phase by means of a (hydrophilic) membrane preventing direct contact of the biocatalyst with the solvent^{4, 12, 29, 63}. However important drawbacks of such systems are membrane fouling due to concentration of biomass and waste products at the membrane surface⁶⁸. This causes decreased mass transfer (decreased extraction of the product) and increased pressure differences that could bring about mixing of the phases^{29, 94}. Thus such process requires periodic cleaning/changing of the system which would in many cases be economically less favourable.

Two-liquid water-solvent systems with direct contact of the phases would be an excellent alternative provided that the biocatalyst is able to cope with the solvent phase. The principle advantage of using solvent-tolerant *P. putida* as a biocatalyst is the new degree of freedom in coping with toxic solvents⁸.

Most conventionally used biocatalysts such as *E. coli* can only withstand solvents that have a $\log P > 5$ sufficiently. For this reason solvents such as octane ($\log P_o/w = 4.6$), dodecane ($\log P_o/w = 6.2$), hexadecane ($\log P_o/w = 8.5$) and bis(2-ethylhexyl)phthalate (BEHP) ($\log P_o/w = 8$) were previously applied in two-liquid water-solvent processes for the production of several hydrophobic epoxides¹¹¹, styrene oxide^{78, 79, 114} and benzaldehydes^{2, 3} with *E. coli* as the biocatalyst. However, these solvents are not necessarily the most appropriate as the physico-chemical nature of many hydroxylated aromatics, aldehydes, alcohols and epoxides are such that they are more effectively removed from the aqueous phase by less lipophilic (more toxic) solvents, with a $\log P_o/w$ between 3 and 5.

In the bioproduction of styrene oxide from styrene in *E. coli* a two-liquid water-BEHP system was applied. From pilot-scale fermentation studies it was concluded that further optimization of styrene oxide production could be reached if styrene concentrations in the system would be higher⁷⁸. This could have been brought about by application of a more polar solvent with lower $\log P_o/w$. However, *E. coli* is not able to tolerate higher styrene concentrations nor such second solvent phase and thus this process appeared to have reached its final limitation. Two possible escape routes were suggested to overcome these problems. The first involved transfer of solvent-tolerant properties from *P. putida* strains to the *E. coli* production strain. The second route involved the use solvent-tolerant *P. putida* as a host for this process⁷⁸. The first option appears to be not realistic in view of the fact that solvent-tolerance in *P. putida* is achieved by various different cellular mechanisms that are inter-linked in a still not clarified fashion. Functional expression in *E. coli* therefore appears far fetched. However, the use of *P. putida* as a host for these types of bioconversions in general appears an attractive concept.

A proof of principle illustrating the versatility of a solvent-tolerant *P. putida* strains in water-solvent systems was demonstrated by the bioconversion of toluene into 3-methylcatechol in a two-liquid water-octanol system^{31, 110}. Such process is not feasible with *E. coli* as the substrate, product and extractant phase are already lethal for this organism in very low concentrations. Octanol was chosen as the extractant phase as its lipophilicity ($\log P_o/w = 2.9$) is closer to that of 3-methylcatechol ($\log P_o/w = 1.5$) compared to commonly used less toxic solvents like octane, dodecane, hexadecane and BEHP.

In a two phase water-octanol system 97% of the 3-methylcatechol and over 99% of the toluene ($\log P_o/w = 2.6$) will partition to the octanol phase. The production of 3-methylcatechol in a 1:1 octanol/aqueous two-phase system was

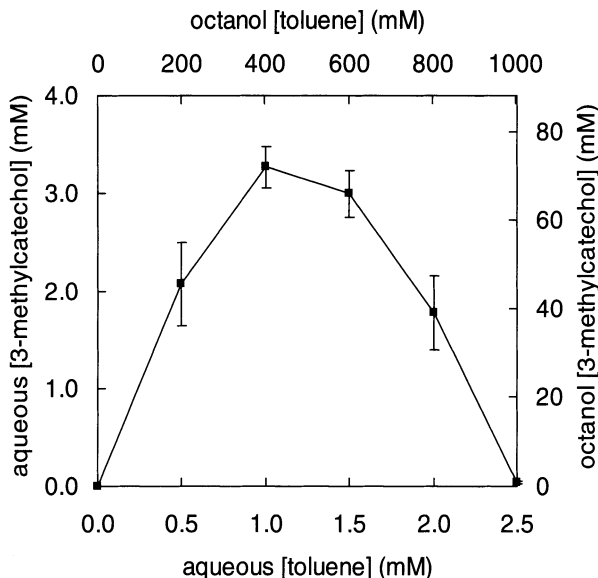


Figure 5. Accumulated aqueous and octanol concentrations of 3-methylcatechol as a function of aqueous and octanol toluene concentration in a 1:1 octanol/aqueous two-phase system. Adapted from Hüsken *et al.*³¹.

monitored at different initial toluene concentrations (Figure 5). It was shown that 3-methylcatechol accumulated in the octanol phase to a concentration of 10 g/L, whereas in a single aqueous system maximally 2 g/L was attained.

5.2. *Pseudomonas* for Green Production of Chemicals

As outlined in the introduction of this chapter there is an increasing drive to develop sustainable production processes in the chemical manufacturing industry. It is expected that whole-cell biocatalysis will be the key process for the production of chemicals from renewable resources such as plant materials (sugar). Already microorganisms are employed for the production of antibiotics, vitamins and amino acids from sugars. For the production of (fine)-chemicals, which are normally not synthesized by the metabolic machinery of micro-organisms green production processes are still at their infancy. However, chemical companies have initiated development of such processes. For example, Dupont and Genencor have announced the development of a bioprocess for the production of 1,3-propanediol from sugars rather than from fossil fuel.

Green chemical production is based on the conversion of central metabolites, such as amino acids, into a given final product via one or two conversions. These extra conversions can be effectuated by the introduction of

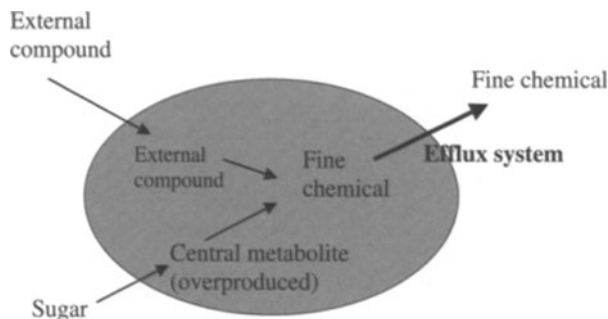


Figure 6. Schematic representation of bioproduction of compounds from an added external compound or from a primary metabolite driven by a solvent efflux system.

appropriate (foreign) genes into the biocatalyst. The production of, for example, aromatics may thus proceed from aromatic amino acids. Cells under normal conditions carefully avoid accumulation of central metabolites. However, the drain caused by the conversion of the metabolite into the final product will keep the metabolic flow running.

In such process it is thus of eminent importance to constantly remove the final product from the cell in order to continuously drive the equilibrium of the reaction towards the product and to pull the relevant metabolic machinery (Figure 6). Here the solvent efflux pumps of *Pseudomonas* strains may play a key role. As outlined in Section 3.3 some of these pumps have been shown to possess a broad substrate specificity ranging from aromatics to alkanes and alcohols. Hence, the solvent efflux pumps of *Pseudomonas* strains not only provide a means to combat toxicity in a given bioconversion process, but could also prove to be excellent drivers of any metabolic chain that converts renewable substrates into (fine-)chemicals.

6. CONCLUSION

An economically feasible biological alternative for the chemical synthesis of many (fine-)chemicals would be highly desirable for the sake of highly specific and sustainable, green, production processes. Bioproduction of many compounds of interest, such as substituted aromatics, aldehydes, alcohols, and epoxides is a constant battle against toxicity. It is increasingly recognized that Pseudomonads, in particular solvent-tolerant *P. putida*, have acquired excellent mechanisms to deal with this toxicity.

Although *P. putida* has long been recognized as a versatile biocatalyst, its application as such is by far not as widespread as compared to *E. coli*.

But in the past few years many constraints with respect to engineering biocatalytic processes based on *P. putida* strains as hosts have been lifted due to the advent of genetic engineering tools and the increasing understanding of metabolic pathways as well as mechanisms of solvent-tolerance.

Recently the genome of *P. putida* KT2440 was annotated, which has revealed its non pathogenic nature⁷⁰. In addition a global view was obtained on the degradation of aromatic compounds by *P. putida*⁴⁰. A genome-scale *in silico* representation of the metabolic and transport network of *P. putida* has been developed on the basis of annotated genome sequence data combined with current biochemical knowledge and physiological information⁶⁰.

These developments are of eminent importance for designing more efficient whole-cell production processes with *Pseudomonas* on centre stage.

In term this will significantly add to the green, economically feasible alternatives for chemical production methods.

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REFERENCES

1. Aono, R. and Kobayashi, H., 1997, Cell surface properties of organic solvent-tolerant mutants of *Escherichia coli* K12. *Appl. Environ. Microbiol.*, 63:3637–3642.
2. Buhler, B., Bollhalder, I., Hauer, B., Witholt, B., and Schmid, A., 2003, Use of the two-liquid phase concept to exploit kinetically controlled multistep biocatalysis. *Biotechnol. Bioeng.*, 20:683–694.
3. Buhler, B., Witholt, B., Hauer, B., and Schmid, A., 2002, Characterization and application of xylene monooxygenase for multistep biocatalysis. *Appl. Environ. Microbiol.*, 68:560–568.
4. Choi, W.J., Choi, C.Y., de Bont, J.A.M., and Weijers, C.A.G.M., 1999, Resolution of 1,2-epoxyhexane by *Rhodotorula glutinis* using a two-phase membrane bioreactor. *Appl. Microbiol. Biotechnol.*, 53:7–11.
5. Collins, A.M., Woodley, J.M., and Lidell, J.M., 1995, Determination of reactor operation for the microbial hydroxylation of toluene in a two-liquid phase process. *J. Ind. Microbiol.*, 14:382–388.
6. Cronan, J.E., 2002, Phospholipid modifications in bacteria. *Curr. Opin. Microbiol.*, 5:202–205.
7. Cruden, D.L., Wolfram, J.H., Rogers, R.D., and Gibson, D.T., 1992, Physiological properties of a *Pseudomonas* strain which grows with *p*-xylene in a two-phase (organic-aqueous) medium. *Appl. Environ. Microbiol.*, 58:2723–2729.
8. De Bont, J.A.M., 1998, Solvent-tolerant bacteria in biocatalysis. *Trends Biotechnol.*, 16:493–499.
9. Dejonghe, W., Boon, N., Seghers, D., Top, E.M., and Verstraete, W., 2001, Bioaugmentation of soils by increasing microbial richness: Missing links. *Environ. Microbiol.*, 3:649–657.
10. Dennis, J.J. and Zylstra, G.J., 1998, Plasposons: Modulair self-cloning minitransposons derivatives for rapid genetic analysis of Gram-negative bacterial genomes. *Appl. Environ. Microbiol.*, 64:2710–2715.

11. Diefenbach, R. and H. Keweloh, 1994, Synthesis of *trans* saturated fatty acids in *Pseudomonas putida* P8 by direct isomerization of the double bond of lipids. *Arch. Microbiol.*, 162:120–125.
12. Doig, S.D., Boam, A.T., Livingston, A.G., and Stuckey, D.C., 1999, Epoxidation of 1,7-octadiene by *Pseudomonas oleovorans* in a membrane bioreactor. *Biotechnol. Bioeng.*, 63:601–611.
13. Duque, E., Segura, A., Mosqueda, G., and Ramos, J.L., 2001, Global and cognate regulators control expression of the organic solvent efflux pumps TtgABC and TtgDEF of *Pseudomonas putida*. *Mol. Microbiol.*, 39:1100–1106.
14. Fleischmann, R.D. *et al.*, 1995, Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science*, 269:496–512.
15. Fukumori, F., Hirayama, H., Takami, H., Inoue, A., and Horikoshi, K., 1998, Isolation and transposon mutagenesis of a *Pseudomonas putida* KT2442 toluene-resistant variant: Involvement of an efflux system in solvent tolerance. *Extremophiles*, 2:395–400.
16. Galbiati, F., Razani, B., and Lisanti, M.P., 2001, Emerging themes in lipid rafts and caveolae. *Cell*, 106: 403–411.
17. Grkovic, S., Brown, M.H., and Skurray, R.A., 2002, Regulation of bacterial drug export systems. *Microbiol. Mol. Biol. Rev.*, 66:671–701.
18. Harrop, A.J., Woodley, J.M., and Lilly, M.D., 1992, Production of naphthalene-*cis*-glycol by *Pseudomonas putida* in the presence of organic solvents. *Enzyme Microb. Technol.*, 14:725–730.
19. Heipieper, H.-J. and de Bont, J.A.M., 1994, Adaptation of *Pseudomonas putida* S12 to ethanol and toluene at the level of fatty acid composition of membranes. *Appl. Environ. Microbiol.*, 60:4440–4444.
20. Heipieper, H.J., Diefenbach, R., and Keweloh, H., 1992, Conversion of *cis* unsaturated fatty acids to *trans*, a possible mechanism for the protection of phenol-degrading *Pseudomonas putida* P8 from substrate toxicity. *Appl. Environ. Microbiol.*, 58:1847–1852.
21. Heipieper, H.-J., Weber, F.J., Sikkema, J., Keweloh, H., and de Bont, J.A.M., 1994, Mechanisms behind resistance of whole cells to toxic organic solvents. *Trends Biotechnol.*, 12:409–415.
22. Held, M., Schmid, A., Kohler, H.-P.E., Suske, W., Witholt, B., and Wubbolts, M.G., 1999, An integrated process for the production of toxic catechols from toxic phenols based on a designer biocatalyst. *Biotechnol. Bioeng.*, 62:641–648.
23. Hiruma, R., Yamaguchi, A., and Sawai, T., 1984, The effect of lipopolysaccharide on lipid bilayer permeability of beta-lactam antibiotics. *FEBS Lett.*, 170:268–272.
24. Holtwick, R., Keweloh, H., and Meinhart, F., 1999, *Cis/trans* isomerization of unsaturated fatty acids of *Pseudomonas putida* P8: Evidence for a heme protein of the cytochrome *c* type. *Appl. Environ. Microbiol.*, 65:2644–2649.
25. Holtwick, R., Meinhart, F., and Keweloh, H., 1997, *Cis-trans* isomerization of unsaturated fatty acids: Cloning and sequencing of the *cti* gene from *Pseudomonas* P8. *Appl. Environ. Microbiol.*, 63:4292–4297.
26. Huertas, M.-J., Duque, E., Marques, S., and Ramos, J.-L., 1998, Survival in soil of different toluene-degrading *Pseudomonas* strains after solvent shock. *Appl. Environ. Microbiol.*, 64:38–42.
27. Hüskens, L.E., 2002, Production of catechols: Microbiology and technology. PhD Thesis, Wageningen University, The Netherlands.
28. Hüskens, L.E., de Bont, J.A.M., Beefink, R., Tramper, J., and Wery, J., 2002, Optimisation of microbial 3-methylcatechol production as affected by culture conditions. *Biocat. Biotrans.*, 20:57–61.
29. Hüskens, L.E., Oomes, M., Schroen, K., Tramper, J., de Bont, J.A.M., and Beefink, R., 2002, Membrane-facilitated bioproduction of 3-methylcatechol in an octanol/water two-phase system. *J. Biotechnol.*, 96:281–289.
30. Hüskens, L.E., Beefink, R., de Bont, J.A.M., and Wery, J., 2001, High-rate 3-methylcatechol production in *Pseudomonas putida* strains by means of a novel expression system. *Appl. Microbiol. Biotechnol.*, 55:571–577.

31. Hüsken, L.E., Dalm, M.C., Tramper, J., Wery, J., de Bont, J.A.M., and Beertink, R., 2001, Integrated bioproduction and extraction of 3-methylcatechol. *J. Biotechnol.*, 88:11–19.
32. Ikura, Y., Yoshida, Y., and Kudo, T., 1997, Physiological properties of two *Pseudomonas mendocina* strains which assimilate styrene in a two-phase (solvent-aqueous) system under static culture conditions. *J. Ferment. Bioeng.*, 83:604–607.
33. Ingram, L.O., 1976, Adaptation of membrane lipids to alcohols. *J. Bacteriol.*, 125:670–678.
34. Ingram, L.O., 1977, Changes in the lipid composition of *Escherichia coli* resulting from growth with organic solvents and with food additives. *Appl. Environ. Microbiol.*, 33:1233–1236.
35. Inoue, A. and Horikoshi, K., 1989, A *Pseudomonas* thrives in high concentration of toluene. *Nature*, 338:264–266.
36. Isken, S. and de Bont, J.A.M., 1996, Active efflux of toluene in a solvent-resistant bacterium. *J. Bacteriol.*, 178:6056–6058.
37. Isken, S. and de Bont, J.A.M., 1998, Bacteria tolerant to organic solvents. *Extremophiles*, 2:229–238.
38. Isken, S. and de Bont, J.A.M., 2000, The solvent efflux system of *Pseudomonas putida* S12 is not involved in antibiotic resistance. *Appl. Microbiol. Biotechnol.*, 54:711–714.
39. Jackson, R.W. and de Moss, J.A., 1965, Effects of toluene on *Escherichia coli*. *J. Bacteriol.*, 90:1420–1425.
40. Jimenez, J.I., Minambres, B., Garcia, J.L., and Diaz, E., 2002, Genomic analysis of the aromatic catabolic pathways from *Pseudomonas putida* KT2440. *Environ. Microbiol.*, 4:824–841.
41. Junker, F. and Ramos, J.L., 1999, Involvement of the cis/trans isomerase Cti in solvent resistance of *Pseudomonas putida* DOT-T1E. *J. Bacteriol.*, 181:5693–5700.
42. Keweloh, H. and Heipieper, H.-J., 1996, Trans unsaturated fatty acids in bacteria. *Lipids*, 31:129–137.
43. Kieboom, J. and de Bont, J.A.M., 2000, Mechanisms of organic solvent tolerance in bacteria. In G. Storz and R. Hengge-Aronis (eds), pp. 393–402. *Bacterial Stress Responses*, American Society for Microbiology, Washington DC.
44. Kieboom, J. and de Bont, J.A.M., 2001, Identification and molecular characterization of an efflux system involved in *Pseudomonas putida* S12 multidrug resistance. *Microbiology*, 147:43–51.
45. Kieboom, J., Dennis, J.J., de Bont, J.A.M., and Zylstra, G.J., 1998, Identification and molecular characterization of an efflux pump involved in *Pseudomonas putida* S12 solvent tolerance. *J. Biol. Chem.*, 273:85–91.
46. Kim, K., Lee, L., Lee, K., and Lim, D., 1998, Isolation and characterization of toluene-sensitive mutants from the toluene-resistant bacterium *Pseudomonas putida* GM73. *J. Bacteriol.*, 180:3692–3696.
47. Kobayashi, H., Takami, H., Hirayama, H., Kobata, K., Usami, R., and Horikoshi, K., 1999, Outer membrane changes in a toluene-sensitive mutant of toluene-tolerant *Pseudomonas putida* IH-2000. *J. Bacteriol.*, 181:4493–4498.
48. Kobayashi, H., Uematsu, K., Hirayama, H., and Horikoshi, K., 2000, Novel toluene elimination system in a toluene-tolerant microorganism. *J. Bacteriol.*, 182:6451–6455.
49. Kobayashi, K., Tsukagoshi, N., and Aono, R., 2001, Suppression of hypersensitivity of *Escherichia coli* acrB mutant to organic solvents by integrational activation of the acrEF operon with the IS1 or IS2 element. *J. Bacteriol.*, 183:2646–2653.
50. Komatsu, T., Moriya, K., and Horikoshi, K., 1994, Preparation of organic solvent-tolerant mutants from *Pseudomonas aeruginosa* strain PAO1161. *Biosci. Biotech. Biochem.*, 58:1754–1755.
51. Koronakis, V., Sharff, A., Koronakis, E., Luisi, B., and Hughes, C., 2000, Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. *Nature*, 405:914–919.
52. Laane, C., Boeren, S., Vos, K., and Veeger, C., 1986, Rules for optimization of biocatalysis in organic solvents. *Biotech. Bioeng.*, 30:81–87.

53. Lau, P.C.K. and Jaworski, J.F., 2003, Industrial sustainability through biotechnology. *ASM News*, 69:110–111.
54. Li, L., Komatsu, T., Inoue, A., and Horikoshi, K., 1995, A toluene-tolerant mutant of *Pseudomonas aeruginosa* lacking the outer membrane protein F. *Biosci. Biotech. Biochem.*, 59:2358–2359.
55. Li, X.-Z., Zhang, L., and Poole, K., 1998, Role of the multidrug efflux systems of *Pseudomonas aeruginosa* in organic solvent tolerance. *J. Bacteriol.*, 180:2987–2991.
56. Ma, D., Alberti, M., Lynch, C., Nikaido, H., and Hearst, J.E., 1996, The local repressor AcrR plays a modulating role in the regulation of *acrAB* genes of *Escherichia coli* by global stress signals. *Mol. Microbiol.*, 19:101–112.
57. Ma, D., Cook, D.N., Hearst, J.E., and Nikaido, H., 1994, Efflux pumps and drug resistance in Gram-negative bacteria. *Trends Microbiol.*, 2:489–493.
58. Ma, D., Cook, D.N., Alberti, M., Pon, N.G., Nikaido, H., and Hearst, J.E., 1995, Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli*. *Mol. Microbiol.*, 16:45–55.
59. Mars, A.E., Gorissen, J.P., van den Beld, I., and Eggink, G., 2001, Bioconversion of limonene to increased concentrations of perillic acid by *Pseudomonas putida* GS1 in a fed-batch reactor. *Appl. Microbiol. Biotechnol.*, 56:101–107.
60. Martins dos Santos, V.A.P., Heim, S., Moore, E.R.B., Strätz, and Timmis, K.N., 2003, *In-silico* analysis of the *Pseudomonas putida* genome: Niche specificity and metabolic versatility. *Environ. Microbiol.*, in press.
61. Matsumoto, M., De Bont, J.A.M., and Isken, S., 2002, Isolation and characterization of the solvent-tolerant *Bacillus cereus* strain R1. *J. Biosci. Bioeng.*, 94:45–51.
62. Miller, E.S. Jr and Peretti, S.W., 2002, Toluene bioconversion to p-hydroxybenzoate by fed-batch cultures of recombinant *Pseudomonas putida*. *Biotechnol. Bioeng.*, 77:340–351.
63. Molinari, F., Aragozzini, F., Cabral, J.M.S., and Prazeres, D.M.F., 1997, Continuous production of isovaldehyde through extractive bio-conversion in a hollow-fiber membrane bioreactor. *Enzyme Microb. Technol.*, 20:604–611.
64. Morita, N., Shibahara, A., Yamamoto, K., Shinkai, K., Kajimoto, G., and Okuyama, H., 1993, Evidence for *cis-trans* isomerization of a double bond in the fatty acids of the psychrophilic bacterium *Vibrio* sp. strain ABE-1. *J. Bacteriol.*, 175:916–918.
65. Moriya, K. and Horikoshi, K., 1993, A benzene-tolerant bacterium utilizing sulfur compounds isolated from deep sea. *J. Ferment. Bioeng.*, 76:397–399.
66. Moriya, K. and Horikoshi, K., 1993, Isolation of a benzene-tolerant bacterium and its hydrocarbon degradation. *J. Ferment. Bioeng.*, 76:168–173.
67. Mosqueda, G. and Ramos, J-L., 2000, A set of genes encoding a second toluene efflux system in *Pseudomonas putida* DOT-T1E is linked to the *tod* genes for toluene metabolism. *J. Bacteriol.*, 182:937–943.
68. Mulder, M., 1996, *Basic Principles of Membrane Technology*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
69. Murakami, S., Nakashima, R., Yamashita, E., and Yamaguchi, A., 2002, Crystal structure of bacterial multidrug efflux transporter AcrB. *Nature*, 419:587–593.
70. Nelson, K., Paulsen, I., Weinel, C., Dodson, R., Hilbert, H., Fouts, D., Gill, S., Pop, M., Martins Dos Santos, V., Holmes, M., Brinkac, L., Beanan, M., DeBoy, R., Daugherty, S., Kolonay, J., Madupu, R., Nelson, W., White, O., Peterson, J., Khouri, H., Hance, I., Lee, P., Holtzapple, E., Scanlan, D., Tran, K., Moazzez, A., Utterback, T., Rizzo, M., Lee, K., Kosack, D., Moestl, D., Wedler, H., Lauber, J., Hoheisel, J., Straetz, M., Heim, S., Kiewitz, C., Eisen, J., Timmis, K., Duesterhoft, A., Tummeler, B., and Fraser, C., 2002, Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ. Microbiol.*, 4:799–808.
71. Nikaido, H. and Zgurskaya, H.I., 2001, AcrAB and related multidrug efflux pumps of *Escherichia coli*. *J. Mol. Microbiol. Biotechnol.*, 3:215–218.

72. Nikaido, H. and Vaara, M., 1985, Molecular basis of bacterial outer membrane permeability. *Microbiol. Rev.* 49:1–32.
73. Ogino, H., Miyamoto, K., and Ishikawa, H., 1994, Organic solvent-tolerant bacterium which secretes an organic solvent-stable lipolytic enzyme. *Appl. Environ. Microbiol.*, 60:3884–3886.
74. Ogino, H., Yasui, K., Shiotani, T., Ishihara, T., and Ishikawa, H., 1995, Organic solvent-tolerant bacterium which secretes and organic solvent-stable proteolytic enzyme. *Appl. Environ. Microbiol.*, 61:4258–4262.
75. Ohta, Y., Maeda, M., Kudo, T., and Horikoshi, K., 1996, Isolation and characterization of solvent-tolerant bacteria which can degrade biphenyl/polychlorinated biphenyls. *J. Gen. Appl. Microbiol.*, 42:349–354.
76. Osborne, S.J., Leaver, J., Turner, M.K., and Dunnill, P., 1990, Correlation of biocatalytic activity in an organic-aqueous two-phase system with solvent concentration in the cell membrane. *Enzyme Microb. Technol.*, 12:281–291.
77. Paje, M.L.F., Neilan, B.A., and Couperwhite, I., 1997, A *Rhodococcus* species that thrives on medium saturated with liquid benzene. *Microbiology*, 143:2975–2981.
78. Panke, S., Held, M., Wubbolts, M.G., Witholt, B., and Schmid, A., 2002, Pilot-scale production of (S)-styrene oxide from styrene by recombinant *Escherichia coli* synthesizing styrene monooxygenase. *Biotechnol. Bioeng.*, 80:33–41.
79. Panke, S., Meyer, A., Huber, C.M., Witholt, B., and Wubbolts, M.G., 1999, An alkane-responsive expression system for the production of fine chemicals. *Appl. Environ. Microbiol.*, 65:2324–2332.
80. Pinkart, H.C. and White, D.C., 1997, Phospholipid biosynthesis and solvent tolerance in *Pseudomonas putida* strains. *J. Bacteriol.*, 179:4219–4226.
81. Pinkart, H.C., Wolfram, J.W., Rogers, R., and White, D.C., 1996, Cell envelope changes in solvent-tolerant and solvent-sensitive *Pseudomonas putida* strains following exposure to *o*-xylene. *Appl. Environ. Microbiol.*, 62:1129–1132.
82. Poole, K., 2001, Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. *J. Mol. Microbiol. Biotechnol.*, 3:255–264.
83. Ramos, J.L., Diaz, E., Dowling, D., de Lorenzo, V., Molin, S., O’Gara, F., Ramos, C., and Timmis, K.N., 1994, The behavior of bacteria designed for biodegradation. *Bio/Technology*, 12:1349–1356.
84. Ramos, J.L., Duque, E., Godoy, P., and Segura, A., 1998, Efflux pumps involved in toluene tolerance in *Pseudomonas putida* DOT-T1E. *J. Bacteriol.*, 180:3323–3329.
85. Ramos, J.L., Duque, E., Huertas, M.J., and Haïdour, A., 1995, Isolation and expansion of the catabolic potential of a *Pseudomonas putida* strain able to grow in the presence of high concentrations of aromatic hydrocarbons. *J. Bacteriol.*, 177:3911–3916.
86. Ramos, J.L., Duque, E., Rodriguez-Herva, J.J., Godoy, P., Haïdour, A., Reyes, F., and Fernandez-Barrero, A., 1997, Mechanisms for solvent tolerance in bacteria. *J. Biol. Chem.*, 272:3887–3890.
87. Ramos, J.L., Duque, E., Gallegos, M.T., Godoy, P., Ramos-Gonzalez, M.I., Rojas, A., Teran, W., and Segura, A., 2002, Mechanisms of solvent tolerance in gram-negative bacteria. *Annu. Rev. Microbiol.*, 56:743–768.
88. Robinson, G.K., Stephens, G.M., Dalton, H., and Geary, P.J., 1992, The production of catechols from benzene and toluene by *Pseudomonas putida* in glucose fed-batch culture. *Biocatalysis*, 6:81–100.
89. Rodriguez-Herva J.J. and Ramos, J.L., 1996, Characterization of an OprL null mutant of *Pseudomonas putida*. *J. Bacteriol.*, 178:5836–5840.
90. Rodriguez-Herva, J.J., Ramos-Gonzales, M.-I., and Ramos, J.L., 1996, The *Pseudomonas putida* peptidoglycan-associated outer membrane lipoprotein is involved in maintenance of the integrity of the cell envelope. *J. Bacteriol.*, 178:1699–1706.

91. Rojas, A., Duque, E., Mosqueda, G., Golden, G., Hurtado, A., Ramos, J.L., and Segura, A., 2001, Three efflux pumps are required to provide efficient tolerance to toluene in *Pseudomonas putida* DOT-T1E. *J. Bacteriol.*, 183:3967–3973.
92. Ronchel, M.C., Ramos, C., Jensen, L.B., Molin, S., and Ramos, J.L., 1995, Construction and behavior of biologically contained bacteria for environmental applications in bioremediation. *Appl. Environ. Microbiol.*, 61:2990–2994.
93. Sanchez-Romero, J.M. and De Lorenzo, V., 1999, Genetic engineering of nonpathogenic *Pseudomonas* strains as biocatalysts for industrial and environmental processes. In A.L. Demain, and J.E. Davies, (eds), *Manual of Industrial Microbiology and Biotechnology*, pp. 460–274. American Society for Microbiology, Washington, DC.
94. Schroën, C.G.P.H., Wijers, M.C., Cohen Stuart, M.A., Van der Padt, A., Van't Riet, K., 1993, Membrane modification to avoid wettability changes due to protein adsorption in an emulsion/membrane bioreactor. *J. Membr. Sci.*, 80:265–274.
95. Schwartz, D.R. and McCoy, C.J., 1977, Epoxidation of 1,7-octadiene by *Pseudomonas oleovorans*: Fermentation in the presence of cyclohexane. *Appl. Environ. Microbiol.*, 34:47–49.
96. Shima, H., Kudo, T., and Horikoshi, K., 1991, Isolation of toluene-resistant mutants from *Pseudomonas putida* PpG1 (ATCC 17453). *Agric. Biol. Chem.*, 55:1197–1199.
97. Shinitsky, M., 1984, Membrane fluidity and cellular functions. In M. Shinitsky, (ed.), *Physiology of Membrane Fluidity*, vol. II. CRC Press, Boca Raton.
98. Sikkema, J., de Bont, J.A.M., and Poolman, B., 1994, Interactions of cyclic hydrocarbons with biological membranes. *J. Biol. Chem.*, 269:8022–8028.
99. Sikkema, J., de Bont, J.A.M., and Poolman, B., 1995, Mechanisms of membrane toxicity of cyclic hydrocarbons. *Microbiol. Rev.*, 59:201–222.
100. Smet de M.J., Kingma, J., and Witholt, B., 1978, The effect of toluene on the structure and permeability of the outer and cytoplasmic membranes of *Escherichia coli*. *Biochim. Biophys. Acta*, 506:64–80.
101. Stover, C.K., Pham, X.-Q.T., Erwin, A.L., Mizoguchi, S.D., Warrenner, P., Hickey, M.J., Brinkman, F.S.L., Hufnagle, W.O., Kowalik, D.J., Lagrou, M., Garber, R.L., Goltry, L., Tolentino, E., Westbrook-Wadman, S., Yuan, Y., Brody, L.L., Coulter, S.N., Folger, K.R., Kas, A., Larbig, K., Lim, R.M., Smith, K.A., Spencer, D.H., Wong, G.K.-S., Wu, Z., Paulsen, I.T., Reizer, J., Saier, M.H., Hancock, R.E.W., Lory, S., and Olson, M.V., 2000, Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature*, 406:959–964.
102. Taylor, S.C., 1985, Biochemical process. US Patent 4,508,822.
103. Tsubata, T. and Kurane, R., 1996, Selection of an *n*-heptanol-resistant bacterium from an organic solvent-sensitive bacterium and characterization of its fatty acids. *Can. J. Microbiol.*, 42:642–646.
104. Vaara, M., Plachy, W.Z., and Nikaido, H., 1990, Partitioning of hydrophobic probes into lipopolysaccharide bilayers. *Biochim. Biophys. Acta*, 1024:152–158.
105. Wackett, L.P., 2003, *Pseudomonas putida*-a versatile biocatalyst. *Nat. Biotechnol.*, 21:136–138.
106. Weber, F.J. and de Bont, J.A.M., 1996, Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes. *Biochim. Biophys. Acta*, 1286:225–245.
107. Weber, F.J., Ooijkaas, L.P., Schemen, R.M.W., Hartmans, S., and de Bont, J.A.M., 1993, Adaptation of *Pseudomonas putida* S12 to high concentrations of styrene and other organic solvents. *Appl. Environ. Microbiol.*, 59:3502–3504.
108. Weber, F.J., Isken, S., and de Bont, J.A.M., 1994, *Cis/trans* isomerization of fatty acids as a defence mechanism of *Pseudomonas putida* strains to toxic concentrations of toluene. *Microbiology*, 140:2013–2017.
109. Wery, J., Hidayat, B., Kieboom, J., and de Bont, J.A.M., 2001, An insertion sequence prepares *Pseudomonas putida* S12 for severe solvent stress. *J. Biol. Chem.*, 276:5700–5706.

110. Wery, J., Mendes da Silva, D.I., and de Bont, J.A.M., 2000, A genetically modified solvent-tolerant bacterium for optimized production of a toxic fine chemical. *Appl. Microbiol. Biotechnol.*, 54:180–185.
111. Witholt, B., de Smet, M.J., Kingma, J., van Beilen, J.B., Kok, M., Lageveen, R.G., and Eggink, G., 1990, Bioconversions of aliphatic compounds by *Pseudomonas oleovorans* in multiphase bioreactors; background and economic potential. *Trend Biotechnol.*, 8:46–52.
112. Woldringh, C.L., 1973, Effects of toluene and phenylethyl alcohol on the ultrastructure of *Escherichia coli*. *J. Bacteriol.*, 114:1359–1361.
113. Woodruff, W.A. and Hancock, R.E.W., 1989, *Pseudomonas aeruginosa* outer membrane protein F: Structural role and relationship to the *Escherichia coli* OmpA protein. *J. Bacteriol.*, 171:3304–3309.
114. Wubbolts, M.G., Favre-Bulle, O., and Witholt, B., 1996, Biosynthesis of synthons in two-liquid-phase media. *Biotechnol. Bioeng.*, 52:301–308.
115. Yoshimura, F. and Nikaido, H., 1982, Permeability of *Pseudomonas aeruginosa* outer membrane to hydrophilic solutes. *J. Bacteriol.*, 152:636–642.
116. Zylstra, G.J. and Gibson, D.T., 1989, Toluene degradation by *Pseudomonas putida* F1. Nucleotide sequence of the *todC1C2BADE* genes and their expression in *Escherichia coli*. *J. Biol. Chem.*, 264:14940–14946.
117. Zylstra, G.J., McCombie, W.R., Gibson, D.T., and Finette, B.A., 1988, Toluene degradation by *Pseudomonas putida* F1: Genetic organization of the *tod* operon. *Appl. Environ. Microbiol.*, 54:1498–1503.

SECONDARY METABOLISM

BIOSYNTHESIS AND REGULATION OF ANTI-FUNGAL METABOLITES BY PSEUDOMONADS

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1. INTRODUCTION

Conventional agriculture relies heavily on the application of agrochemical inputs, including herbicides and fungicides to maintain consistent high yields and to protect crops against pathogens. Nevertheless, despite the widespread use of agrochemicals, increased awareness of possible adverse effects on human health and the environment has fuelled the desire for more sustainable alternatives to conventional agricultural practices. Reducing chemical inputs in agriculture requires, however, the availability of realistic alternatives for the control of plant pathogens. It has long been recognised that some naturally occurring bacteria and fungi are antagonistic to crop pathogens and may provide such alternative approaches. Utilisation of microbial inoculants for sustainable agri-industrial applications such as phytostimulation, biofertilisation, bioremediation and biocontrol has been the subject of a number of recent reviews^{9, 23, 43, 69, 112, 114}. For a biocontrol strain to be successful, there are certain requirements that need to be fulfilled. These include the ability to

survive in the rhizosphere and to compete against the resident microbial populations, as well as providing protection for the plant against pathogens at the time and site of infection¹⁸. Characteristics of the soil-borne fluorescent *Pseudomonads* have highlighted this group of rhizobacteria as ideal candidates for use in biocontrol applications^{9, 43, 114}. Despite the intrinsic suitability of a number of these microbial strains, there are some limitations that have restricted their success as commercial alternatives to fungicides. A major limitation has been difficulty in transferring successful microcosm and greenhouse results into field situations. Variable efficacy in the field can be due to external factors such as particular soil, climatic, crop or pathogen conditions, but it can also be a consequence of traits that are intrinsic to the bacterial strain. These include reduced ecological fitness and poor colonisation efficacy in certain agricultural systems, and inadequate production of required antifungal metabolites at the time and concentration they are required to control disease. It is clear from work to date that a better understanding of the biochemistry and ecology of biocontrol strains is central to overcoming these obstacles to create effective strains for field applications.

Three major mechanisms by which *Pseudomonas* strains control phytopathogenic fungi have been put forward. First, because of their broad metabolic diversity and ability to colonise the rhizosphere in high numbers, *Pseudomonads* are very effective competitors for nutrients in the rhizosphere. This can provide a competitive edge over many pathogens and can lead to niche-exclusion of some phytopathogens. Another significant advantage comes from the ability of *Pseudomonads* to compete very effectively for iron, which is generally limited in soils. In the *Pseudomonads*, sophisticated and diverse iron uptake systems exist, with the majority of strains producing complex peptidic siderophores, namely pyoverdines or pseudobactins^{22, 87}. These siderophores bind ferric iron and the resulting complexes are recognised and taken up by specific outer membrane receptors⁷⁰. *Pseudomonas* siderophores have been implicated in the ability of these bacteria to colonise diverse ecological niches and to compete with pathogens for iron. The second mechanism relies on the fact that some strains, without causing disease themselves, activate plant defences against disease, leading to a state known as induced systemic resistance (ISR) in the plant^{4, 106, 109, 111}. Plants in which ISR has been triggered are intrinsically more resistant to pathogen attack. The detailed mechanism by which ISR comes about remains to be fully determined, though there has been a report implicating siderophores in the process⁴. In the case of the third mechanism, many *Pseudomonas* strains produce extracellular enzymes and metabolites that are directly antagonistic to fungal pathogens. The diversity of antifungal metabolites produced by *Pseudomonas* biocontrol strains is illustrated in Table 1. Phenazines and 2,4-diacetylphloroglucinol (PHL)

Table 1. Antifungal metabolites produced by *Pseudomonas* spp.

Antifungal metabolite	Biocontrol species/ strain	Target pathogen	Host plant/location	References
2,4-diacetylphloroglucinol (Phl)	<i>Pseudomonas fluorescens</i> F113 CHAO	<i>Phytophthora ultimum</i>	Sugarbeet, Ireland	Shanahan <i>et al.</i> , 1992 ⁹⁹
		<i>Gaeumannomyces graminis</i> var <i>tritici</i> , <i>P. ultimum</i> , <i>Thielaviopsis basicola</i>	Tobacco, Switzerland	Laville <i>et al.</i> , 1992 ⁵⁸
	Q2-87	<i>G. graminis</i> var <i>tritici</i>	Wheat, WA, USA	Vincent <i>et al.</i> , 1991 ¹¹³
	Pf-5	<i>P. ultimum</i> , <i>Rhizoctonia solani</i>	Cotton, Texas, USA	Howell and Stipanovic, 1980
Phenazine derivatives	Q8r1-96	<i>G. graminis</i> var <i>tritici</i>	Wheat, WA, USA	Raaijmakers and Weller, 2001 ⁸⁹
	<i>P. chlororaphis</i> PCL 1391	<i>Fusarium oxysporum</i>	Tomato, Spain	Chin-A-Woeng <i>et al.</i> , 1998 ²⁰
	<i>P. aureofaciens</i> 30-84	<i>G. graminis</i> var <i>tritici</i>	Wheat, Kansas, USA	Pierson and Thomashow, 1992 ⁸⁶
Butyrolactones	<i>P. fluorescens</i> 2-79	<i>G. graminis</i> var <i>tritici</i>	Wheat, WA, USA	Gurusiddaiah <i>et al.</i> , 1986
	<i>P. aureofaciens</i> 63-28	<i>P. ultimum</i> , <i>Phytophthora cryptogea</i>	Canola, Canada	Garnard <i>et al.</i> , 1997 ³⁹
Oomycin A Aerugine	<i>P. fluorescens</i> Hv37a	<i>P. ultimum</i>	Barley, USA	Gutterson <i>et al.</i> , 1986 ⁴²
	<i>P. fluorescens</i> MM-B16	<i>Colletotrichum orbiculare</i> , <i>Phytophthora capsici</i>	Mountain forest soil, Korea	Lee <i>et al.</i> , 2003 ⁵⁹

Table 1. Continued

Antifungal metabolite	Biocontrol species/strain	Target pathogen	Host plant/location	References
<i>N</i> -mercapto-4-4-formylcarbostyryl Pyoluteorin	<i>P. fluorescens</i> G308	<i>F. oxysporum</i> ,	Barley leaves, Germany	Fakhouri <i>et al.</i> , 2001 ³³
	<i>P. fluorescens</i> Pf-5	<i>F. culmorum</i> <i>P. ultimum</i> , <i>R. solani</i>	Cotton, Texas, USA	Howell and Stipanovic, 1980
	<i>P. fluorescens</i> CHAO	<i>Thielaviopsis basicola</i> ,	Tobacco, Switzerland	Laville <i>et al.</i> , 1992 ⁵⁸
Pyrrolnitrin	<i>P. fluorescens</i> BL915	<i>P. ultimum</i> <i>R. solani</i>	Cotton, USA	Van Pee and Ligon 2000 ¹¹⁰
Viscosinamide	<i>P. fluorescens</i> DR54	<i>P. ultimum</i> ,	Sugarbeet, Denmark	Nielsen <i>et al.</i> , 1999 ⁷³
Tensin	<i>P. fluorescens</i> 96.578	<i>R. solani</i>	Sugarbeet, Denmark	Nielson <i>et al.</i> , 2000 ⁷⁴
	<i>Pseudomonas</i> sp. DSS73	<i>R. solani</i>		Sorensen <i>et al.</i> , 2001 ¹⁰²
Pseudophomins B	<i>P. fluorescens</i> BRG100	<i>Phoma lingam</i> , <i>Sclerotinia sclerotiorum</i>	Canada	Pedras <i>et al.</i> , 2003 ⁷⁷

Adapted from ref. [87].

are the most frequently exploited classes of antifungal metabolites in *Pseudomonads*, and these have been extensively studied in the context of bio-control. More recently, lipodepsipeptides, a novel class of metabolites involved in *Pseudomonas* mediated antagonism against fungal pathogens, have been identified. On a comparative scale, however, less information about these molecules is available at this stage.

The aim of this chapter is to provide an overview of recent developments regarding the biosynthesis and regulation of PHL and phenazines in *Pseudomonas* biocontrol strains. These metabolites are produced by many antagonistic strains, and PHL production in particular, has long been associated with naturally disease-suppressive soils^{90, 116}. The application of recombinant DNA technology has been an important tool in establishing irrefutable direct links between biocontrol efficacy of soil-borne *Pseudomonads* and production of antifungal metabolites^{14, 20, 34, 54, 88}. It is now possible with advances in biochemical and functional genomic-based technologies to develop strategies to improve antifungal metabolite production in *Pseudomonas* biocontrol strains. These include reprogramming genetic regulatory circuits governing antifungal metabolite biosynthesis. Functional genomic approaches to enhancing antifungal metabolite production are being further facilitated by the release of full draft and finished genome sequences of a number of *Pseudomonas* strains namely, *Pseudomonas fluorescens* strains Pf-01 (JGC), Pf-5 (TIGR) and SBW25 (Sanger Centre), *Pseudomonas putida* strain KT2440^{71, 72}, *Pseudomonas syringae* (TIGR) and *Pseudomonas aeruginosa* strain PAO1¹⁰³. By understanding the mechanisms regulating biosynthesis of PHL and phenazines, progress can be made towards the development of a new generation of microbial inoculants that may be realistic alternatives to fungicides.

2.1. Structure and Biosynthesis of Phenazines

Phenazine compounds encompass a large family of heterocyclic nitrogen-containing compounds produced almost exclusively by bacteria of the genera *Pseudomonas*, *Streptomyces*, *Nocardia*, *Sorangium*, *Brevibacterium* and *Burkholderia*¹⁰⁸. There are currently over 50 known phenazine compounds with the same basic structure, differing only in the derivatisation of the heterocyclic core. These modifications largely determine the physical properties of phenazines and influence their biological activity against plant and animal pathogens. The more common phenazines produced by *Pseudomonads* are illustrated in Figure 1. Phenazine compounds possess a broad range of biological activity against fungi and other bacteria. Although this activity is not entirely understood, it is thought that phenazines diffuse across the membrane and undergo redox cycling in the presence of reducing agents and molecular oxygen resulting in the accumulation of toxic superoxide ($O_2^{\cdot-}$) and hydrogen

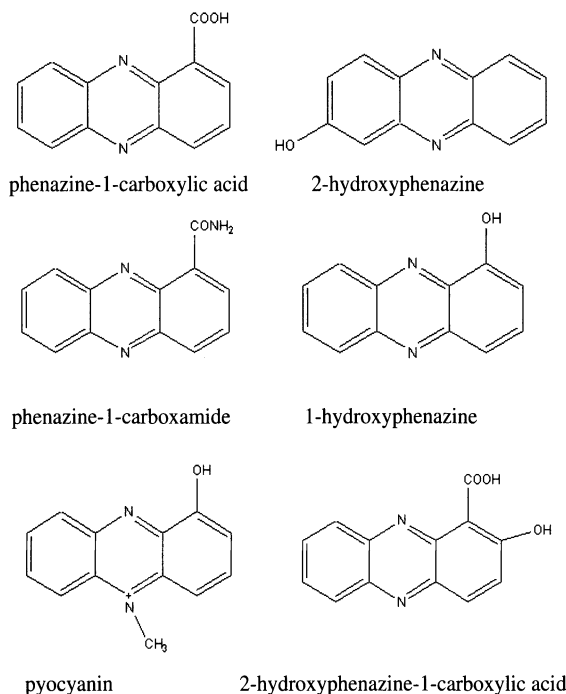


Figure 1. Structures of phenazines produced by *Pseudomonads*. The phenazine derivatives that have been commonly detected in *Pseudomonas* species are shown. *P. fluorescens* 2-79 produces phenazine-1-carboxylic acid. *P. aeruginosa* PAO1 is known to produce 1-hydroxy-5-methylphenazinium betaine (pyocyanin), phenazine-1-carboxamide and 1-hydroxyphenazine. *P. aureofaciens* 30-84 produces 2-hydroxyphenazine, 2-hydroxyphenazine-1-carboxylic acid and phenazine-1-carboxylic acid. *Pseudomonas chlororaphis* PCL1391 produces phenazine-1-carboxamide. For clarity, the species name *P. aureofaciens* is used throughout this chapter to refer to the specific strain *P. aureofaciens* 30-84, although *P. aureofaciens* is now reclassified as part of the *P. chlororaphis* lineage.

peroxide (H_2O_2). This overwhelms the cellular superoxide dismutases of the cell and eventually leads to cell injury and death²⁴. Tolerance of *Pseudomonads* to their own phenazines may be mediated by cellular superoxide dismutases, and there are some data linking activity of superoxide dismutases and phenazine production in *P. aeruginosa*^{45–47}.

Early studies with radiolabelled precursors revealed links between phenazine biosynthesis and the shikimic acid pathway in several microorganisms¹⁰⁸. This pathway is responsible for synthesis of the aromatic amino acids as well as a range of secondary metabolites in bacteria (Figure 2). Based on structural and functional analyses, phenazine molecules are products of the common bacterial aromatic acid pathway, in which chorismate is the probable

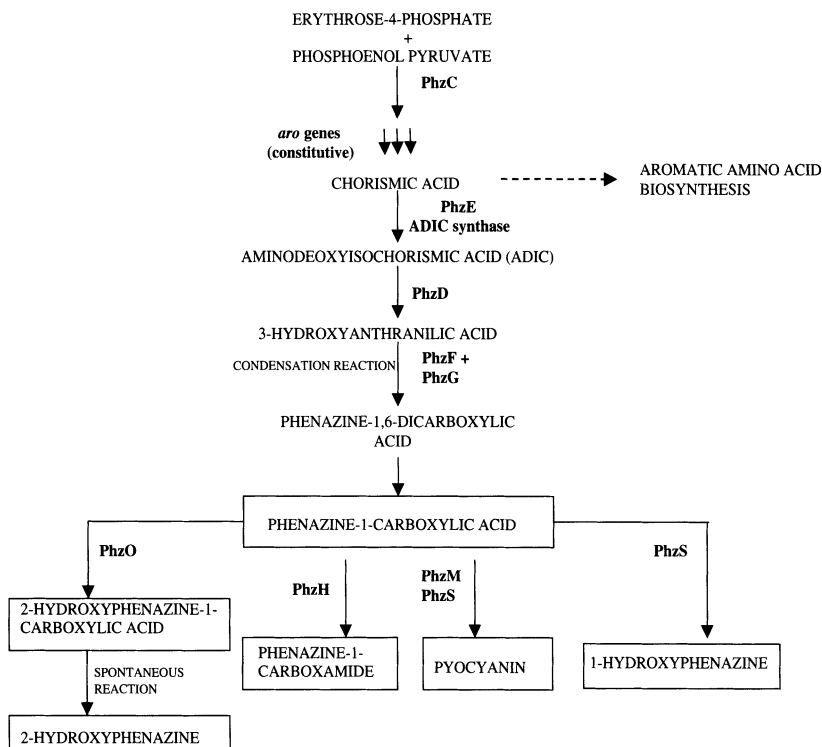


Figure 2. Pathway for phenazine biosynthesis. Phenazines are products of the bacterial common aromatic amino acid pathway, with chorismic acid as the probable branch point intermediate. PhzC increases flux into the shikimic acid pathway to provide chorismic acid for phenazine biosynthesis. Other proteins involved in synthesis of chorismate are constitutively expressed. The proteins involved in the conversion of chorismate to phenazine-1,6-dicarboxylic acid are indicated. The mechanism of subsequent modification to phenazine-1-carboxylic acid (PCA) is not known. PCA is an endpoint in some species but in others it can be further modified to form different phenazine molecules as indicated. *P. aureofaciens* 30-84 produces phenazine-1-carboxylic acid and, by the action of PhzO, 2-hydroxyphenazine-1-carboxylic acid. 2-hydroxyphenazine is also produced spontaneously from 2-hydroxyphenazine-1-carboxylic acid in this bacterium. Phenazine-1-carboxamide, produced by both *P. aeruginosa* and *P. chlororaphis*, is produced from phenazine-1-carboxylic acid by the action of the PhzH protein. *P. aeruginosa* also possesses PhzS, which is responsible for the production of 1-hydroxyphenazine and, in concert with PhzM, pyocyanin. End products of phenazine biosynthesis are boxed.

branch point intermediate⁶¹. Two molecules of chorismic acid are believed to form the phenazine nucleus via a condensation reaction^{50, 51} although the precise chemistry remains to be elucidated. A number of other intermediates are produced, culminating in the formation of phenazine carboxylic acid (PCA)⁶⁰. In some bacteria, other phenazines are subsequently derived from PCA.

2.2. Genetics and Biochemistry of Phenazine Biosynthesis

Fluorescent *Pseudomonas* spp. are, to date, the only microorganisms for which the genes responsible for the assembly of the heterocyclic phenazine nucleus have been cloned and sequenced^{64, 65, 84, 86}. The core phenazine locus, which is responsible for phenazine-1-carboxylic acid (PCA) synthesis, is highly conserved among fluorescent *Pseudomonas* spp. (Figure 3). Depending on the strain or species, additional genes may be present that encode specific enzymes that modify PCA to yield a variety of different phenazine products. The genes involved in phenazine biosynthesis in *Pseudomonas aureofaciens* 30-84 were the first to be cloned and sequenced and termed *phzXYFABCD*^{84, 86}. In other phenazine producing *Pseudomonads*, this operon is termed *phzABCDEFGG* and, except when discussing *P. aureofaciens* 30-84, this nomenclature will be used throughout this chapter.

The proposed biochemical pathway for synthesis of the major phenazines in *Pseudomonads* is depicted in Figure 3. This is based on a study of the phenazine biosynthetic operon in *P. fluorescens* 2-79 carried out by Mavrodi and co-workers⁶⁵. The predicted amino acid sequence of PhzC shares similarity with 3-deoxy-D-arabino-heptulasonate-7-phosphate synthases (DAHP synthases), which catalyse the first reaction in the shikimic acid pathway, the condensation of erythrose-4-phosphate and phosphoenol pyruvate¹³. *phzC* is expressed in late growth phase and may increase substrate flow into the shikimic acid pathway to ensure sufficient chorismic acid for PCA production. The remainder of the enzymes needed for chorismate biosynthesis are

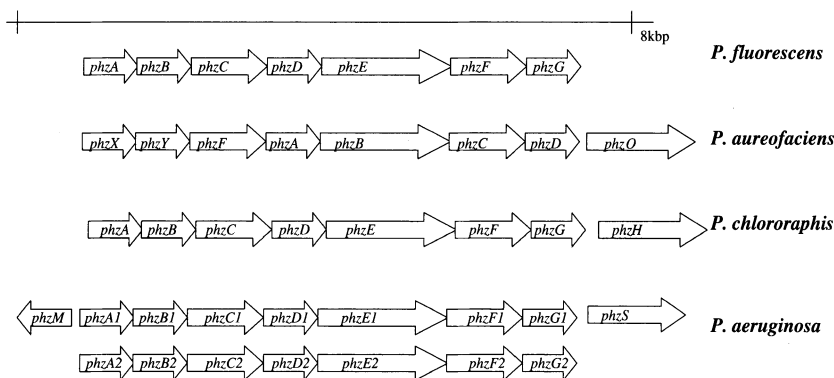


Figure 3. Organisation of the phenazine biosynthetic operon. Schematic representation of the phenazine biosynthetic operon in selected fluorescent *Pseudomonas* spp. The core seven gene operon is conserved in all phenazine-producing species of *Pseudomonas* in which the biosynthetic genes have been characterised. Additional genes providing further functionality and regulation are present in some strains as indicated (adapted from ref. [21]).

probably encoded by the constitutively expressed genes *aroD*, *aroB*, *aroE*, *aroL*, *aroA* and *aroC*. The PhzE protein is most closely related to anthranilate synthase (AS) enzymes involved in biosynthesis of tryptophan and it has been postulated that this protein functions as an aminodeoxyisochorismate (ADIC) synthase to form ADIC from chorismate. PhzD shares sequence similarity with 2,3-dihydro-2,3 dihydroxybenzoate synthases, including the *E. coli entB* gene which catalyses the hydrolysis of pyruvate from isochorismic acid³¹, suggesting that, PhzD may remove the pyruvate side-chain from ADIC to yield 3-hydroxyanthranilic acid. The *phzF* and *phzG* gene products are believed to catalyse the condensation of two molecules of 2-hydroxyanthranilic acid to generate phenazine-1,6-dicarboxylic acid from which PCA is derived, although the details of that final reaction remain to be elucidated. The role played by the *phzA* (*phzX*) and *phzB* (*phzY*) gene products is as yet unclear. These genes, which are conserved in all phenazine loci, are remarkably similar to each other, suggesting that they may have evolved from a gene duplication event⁶⁵. Functional analyses indicate that these genes may influence the type and relative amounts of aromatic and heterocyclic phenazine compounds synthesised and may also function to stabilise a postulated multi-enzyme phenazine biosynthetic complex. In the absence of these two genes, the biosynthetic system functions, but the specificity and efficiency of phenazine biosynthesis decreases dramatically⁶⁵.

The core pathway responsible for PCA synthesis is conserved among fluorescent *Pseudomonads*. Specific strains carry additional genes that encode enzymes that further modify PCA to yield a variety of different phenazine products (Figure 3). *P. aureofaciens* 30-84 contains a gene designated *phzO* located downstream from the core phenazine operon that encodes a 55 kDa aromatic mono-oxygenase²⁴. PhzO is responsible for the hydroxylation of phenazine-1-carboxylic acid to produce 2-hydroxyphenazine-1-carboxylic acid by facilitating the addition of a hydroxyl group to PCA at the ortho position relative to the carboxyl group. It is thought that the subsequent decarboxylation of 2-hydroxyphenazine-1-carboxylic acid to 2-hydroxyphenazine occurs spontaneously. A mutant of *P. aureofaciens* 30-84 inactivated in *phzO* produces only phenazine-1-carboxylic acid²⁴.

Pseudomonas chlororaphis PCL1391 controls tomato foot and root rot caused by *Fusarium oxysporum* f.sp. *radicis lycopersica*. The biocontrol activity associated with this bacterium is mediated by the phenazine compound phenazine-1-carboxamide (PCN)²⁰. In *P. chlororaphis*, the *phzH* gene lies downstream of the *phzA-G* operon²¹. A *phzH* mutation results in a strain unable to produce phenazine-1-carboxamide, and instead produces the precursor PCA in amounts similar to those of phenazine-1-carboxamide produced by wild-type strains²¹. The *phzH* gene product has similarity to asparagine synthases, which catalyse the transfer of the amido nitrogen of glutamine to

aspartate to produce glutamate and asparagine⁶². This similarity and the accumulation of phenazine-1-carboxylic acid in a *phzH* mutant suggest that PhzH functions to convert the carboxylic moiety of phenazine-1-carboxylic acid to the 1-carboxamide group. This conversion may influence the biocontrol ability of *P. chlororaphis* strains since the *phzH* mutant appears to lose its biocontrol ability against *Fusarium*²¹.

Strains of *P. aeruginosa* produce a variety of redox-active phenazine compounds including pyocyanin (1-hydroxy-5-methylphenazinium betaine), phenazine-1-carboxylic acid, 1-hydroxyphenazine and phenazine-1-carboxamide^{12, 108}. Ninety to ninety-five per cent of *P. aeruginosa* isolates produce pyocyanin, a compound which is an important virulence factor in this opportunistic pathogen^{28, 29, 53, 57}. Two independent functional phenazine biosynthetic loci that are homologous to those of other Pseudomonads have been cloned from *P. aeruginosa* PAO1 (Figure 3)⁶⁴. The conversion of PCA to pyocyanin is mediated by products of two genes termed *phzM* and *phzS* (Figure 2). *phzM* encodes a putative phenazine-specific methyl transferase, and *phzS* encodes a flavin-containing mono-oxygenase⁶⁴. A third gene designated *phzH*, which is a homologue of *phzH* in *P. chlororaphis* PCL1391, controls synthesis of phenazine-1-carboxamide from PCA⁶⁴. In *P. aeruginosa* PAO1, the *phzM* and *phzS* genes are located flanking one of the core biosynthetic operons (Figure 3), whereas the *phzH* gene is distally located.

3.1. Structure and Biosynthesis of 2,4-Diacetylphloroglucinol

The natural products monoacetylphloroglucinol (MAPG) and 2,4-diacetylphloroglucinol (DAPG/PHL) are produced by many fluorescent *Pseudomonas* spp. of diverse geographical origins⁵⁴, though synthesis has been best studied in *P. fluorescens*. These two molecules consist of a conjugated aromatic ring with either an acetyl group located at position 2 in the case of MAPG, or at positions 2 and 4 in the case of PHL (Figure 4A). The alternating positions of the hydroxyl groups on the benzenic ring intuitively suggest that the MAPG is synthesised *via* a polyketide pathway⁹⁹. MAPG is believed to be synthesised by the sequential condensation of one molecule of malonyl-CoA with three molecules of acetyl-CoA, though the precise chemistry has not been determined. MAPG is a precursor molecule and is subsequently converted to PHL by the addition of a further acetyl group⁹⁹. The kinetics of PHL production are interesting, with maximum production occurring during late logarithmic/early stationary phase (Figure 4B). Following peak production, PHL levels fall rapidly, indicating that PHL is either degraded (possibly via MAPG) or further processed into a more complex molecule. There is limited

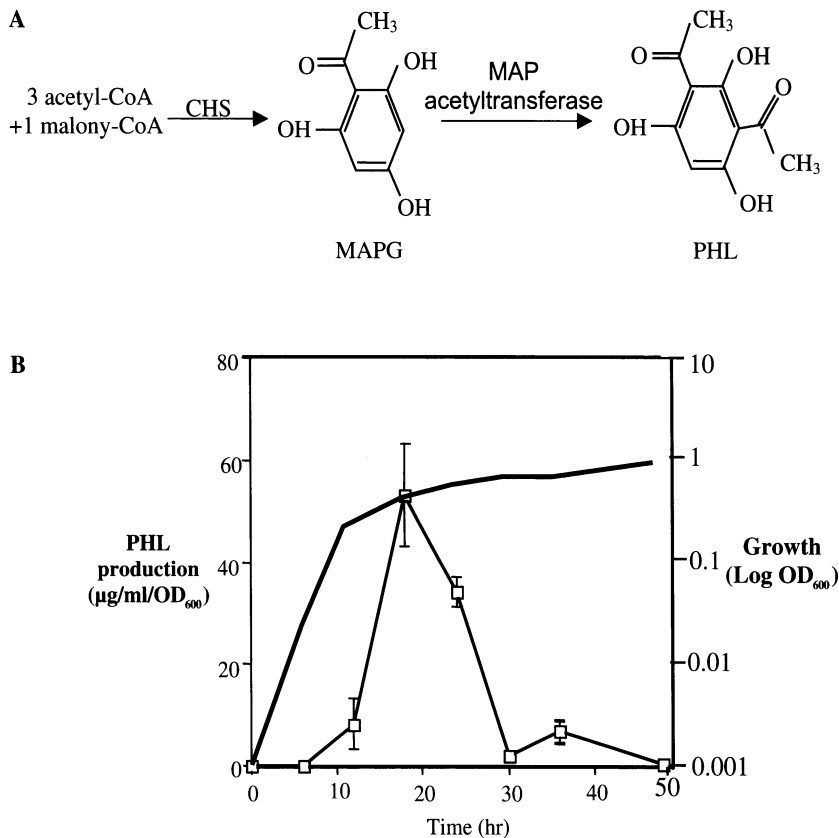


Figure 4. Synthesis of phloroglucinol by *P. fluorescens*. (A) Proposed biosynthetic pathway for phloroglucinol. PHL is believed to be synthesised from the precursors acetyl-CoA and malonyl-CoA via the intermediate MAPG. The precise biochemistry is not known, however, the putative enzyme activities are indicated. (B) Kinetics of Phl production by *P. fluorescens* F113. Phl production by a culture growing in minimal medium is shown. The level of PHL in the growth media reaches a maximum early in stationary phase and drops sharply thereafter.

information currently available on the fate of PHL, and on the kinetics of production *in situ* in the rhizosphere, though it can be detected in that environment¹⁰. Although PHL is known to have activity against a broad spectrum of target organisms, there is little information on its mode of action. It appears not to possess membraneolytic activity and there have been suggestions that PHL may impair electron transport systems but no specific mechanisms of action have been proposed. The mechanism of resistance of producing strains is also not known, but it is believed that export of PHL by the PhlE permease probably contributes to this resistance (see Section 3.2).

3.2. Genetics and Biochemistry of PHL Biosynthesis

The genes involved in the biosynthesis of PHL have been characterised from several *Pseudomonas* strains. Genetic and sequence data show that the *phl* locus from *P. fluorescens* F113 and Q2-87 is located on a genomic DNA region comprising a number of functionally linked transcriptional units^{7, 26}. Although sequence of the full operon is not available from the strain *P. fluorescens* CHAO, partial sequence indicates a similar genetic organisation probably occurs in this strain⁹⁷. Thus, it is believed that the organisation of the locus, comprising both structural and regulatory genes, is conserved in PHL-producing *Pseudomonads* (Figure 5). Although none of the structural proteins has been characterised biochemically, functions have been ascribed to these proteins based on genetic studies and sequence analysis.

The structural genes, *phlA*, *phlC*, *phlB* and *phlD* are believed to be transcribed as a single operon, *phlACBD*⁷. It remains a possibility, however, that *phlD* is also transcribed independently as the *phlB-phlD* intergenic region is approximately 150 bp long and has motifs bearing similarity with σ^{70} consensus recognition sequences. These potential DNA polymerase binding sites are present in *P. fluorescens* strains F113 and Q2-87. *phlD* gene encodes a predicted protein of 349 amino acids and is highly conserved (81–97%) between the *Pseudomonas* spp. for which full-length sequence is available. PhlD presents striking homology with chalcone and stilbene synthases (CHS/STS) from plants and based on its sequence can be classified as a type III polyketide synthase^{7, 91}. Plant CHS enzymes function as homodimers and catalyse

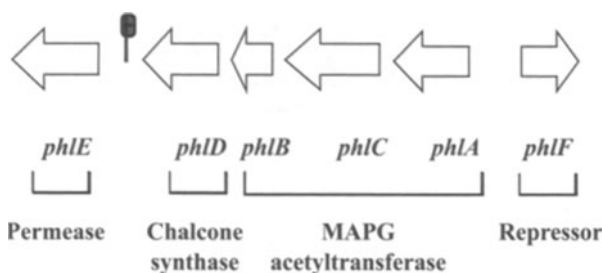


Figure 5. Organisation of the PHL biosynthetic operon. The major genes of the *phl* biosynthetic operon have been cloned from *P. fluorescens* F113 and Q2-87. The putative function that has been assigned to each open reading frame is indicated. Heterologous expression studies have shown that *phlA-phlD* encode the proteins required for synthesis of PHL. *phlE* is believed to encode a permease and *phlF* a transcriptional repressor. Only in the case of PhlF, has biochemical data confirmed the functional assignment. In *P. fluorescens* CHAO, two putative genes *phlG* and *phlH*, were identified 3' (downstream) of *phlF* (not shown), though the roles played by the proteins encoded by these genes is not known.

both condensation and cyclisation steps, with malonyl-CoA constituting the extender unit in these reactions¹¹⁸. This would suggest that PhlD catalyses the condensation of linear primer molecules such as malonyl-CoA and acetyl-CoA, to form MAPG (Figure 4)^{7, 91}. PhlA, PhlC and PhlB are believed to catalyse the transacetylation of MAPG to PHL (Figure 4). Mutation of any one of these genes abolishes both MAPG production and in vitro transacetylation activity, suggesting that these proteins may function collectively⁷. The intergenic regions *phlA-phlC* and *phlC-phlB* are very short and do not contain known sigma factors recognition sequences supporting the experimental data that *phlA*, *phlC* and *phlB* are transcribed as single polycistronic RNA. The *phlA* promoter has been studied experimentally in *P. fluorescens* F113 and is located some 363 base pairs upstream of the ATG translational start in this strain. Putative σ^{70} -10 and -35 elements directing transcription are conserved in *P. fluorescens* strains F113, Q2-87 and CHA0². The *phlA* gene encodes a protein with a predicted molecular mass of approximately 38 kDa. Database searches indicate that PhlA shows some homology to β -ketoacyl-acyl-carrier protein synthases III from Archaea (FabH; 36%) and Eubacteria (FabH; 19%). These proteins are involved in fatty acid production and, in *E. coli*, FabH catalyses both condensation and transacetylation reactions involved in the initiation of fatty acid biosynthesis¹⁰⁷. The sequence similarity of PhlA with FabH may indicate that PhlA possesses transacetylation activity. *phlC* encodes a predicted protein of 398 amino acids that shows 28% identity to the N-terminal thiolase domain of rat carrier protein-x (SCP-x)⁹⁸. The similarity of PhlC with the entire length of the thiolase domain of SCP-x and the conservation of residues at the putative active site suggest that the two proteins may have similar conformational features that could facilitate an interaction between PhlC and MAPG in a manner analogous to that between SCP-x and its sterol ligand. The *phlB* gene encodes a small protein of 146 amino acids and shares 34% identity with the nucleic acid-binding protein AcaC of *Pyrococcus furiosus* (Acc no. Q51796). By analogy with gene clusters involved in fatty acid biosynthesis, the position of the *phlB* gene downstream of β -ketoacyl synthase and thiolase genes suggests that it may encode an acyl carrier protein (ACP), though this has not been demonstrated experimentally.

The *phlE* gene is located downstream of the *phlACBD* operon. A strong Rho-dependent transcription terminator sequence immediately follows the *phlD* translation stop codon indicating that *phlE* is transcriptionally independent of *phlACBD*. The *phlE* gene encodes a protein of 423 amino acids that shows clear homology with proteins in the major facilitator superfamily (MFS) of membrane transporters (Abbas and O'Gara, unpublished)⁷. Hydrophathy analysis predicts that PhlE is organised in two sets of six hydrophobic α -helices, of 23 aa each, separated by a central hydrophilic loop, consistent with the 12 transmembrane helices found in this family of proteins.

The location of *phlE* in the *phl* locus and the predicted secondary structure of the PhlE protein suggest a role for PhlE in the efflux of PHL from the bacterial cell. *phlE* null mutants of *P. fluorescens* strains F113 and Q2-87 produce less total PHL than the wild-type parents, but PHL export is only reduced by approximately 50% suggesting that PhlE is not the only export route for PHL from the cell (Abbas and O’Gara, unpublished)⁶. There are indications suggesting a wider role for PhlE in *P. fluorescens* beyond its proposed action as a PHL efflux system. PhlE is implicated in resistance and tolerance of *P. fluorescens* to a range of environmental stresses including high exogenous levels of PHL, high osmolarity, oxidative stress and heat shock (Abbas and O’Gara, unpublished). Further work is required to determine the mechanism of PHL transport and the role of PhlE in PHL production and stress tolerance, functions that are important contributors to the biocontrol ability of *P. fluorescens* F113.

4.1. Regulation of Secondary Metabolite Production

Precise regulation of gene expression in response to changing environmental conditions is necessary for survival of microbial populations. Accordingly, production of secondary metabolites in *Pseudomonas* is tightly controlled at the genetic level. A variety of regulatory systems play a part in controlling the timing and level of production of phenazines and phloroglucinol in *Pseudomonads*. These include global regulatory systems that control a large number of genes as well as pathway-specific mechanisms that are particular to the biosynthetic genes directly involved in secondary metabolite production. The repertoire of control systems enable the producing bacterium to respond to general environmental conditions as well as to specific conditions of particular relevance to metabolite production. Regulation has been shown to operate at both the transcriptional and post-transcriptional levels, and this obviously creates an enhanced capacity of the bacterium to react quickly to changing environmental conditions.

4.2. Regulation of PHL Production by the Transcriptional Repressor PhlF

It is well established that the *phlF* gene, which is divergently transcribed from *phlA* (Figure 4), encodes a pathway-specific transcriptional repressor, PhlF^{2, 25, 97}. PhlF shows strong homology with other helix-turn-helix transcriptional repressors, particularly in the DNA binding region (Figure 6A). The *phlF* and *phlA* genes display opposite expression profiles during growth of *P. fluorescens*². In early to mid logarithmic phase, *phlF* expression is high and *phlA* low, whereas in late logarithmic and early stationary phase, *phlF* expression falls and *phlA* expression reaches its maximum level.

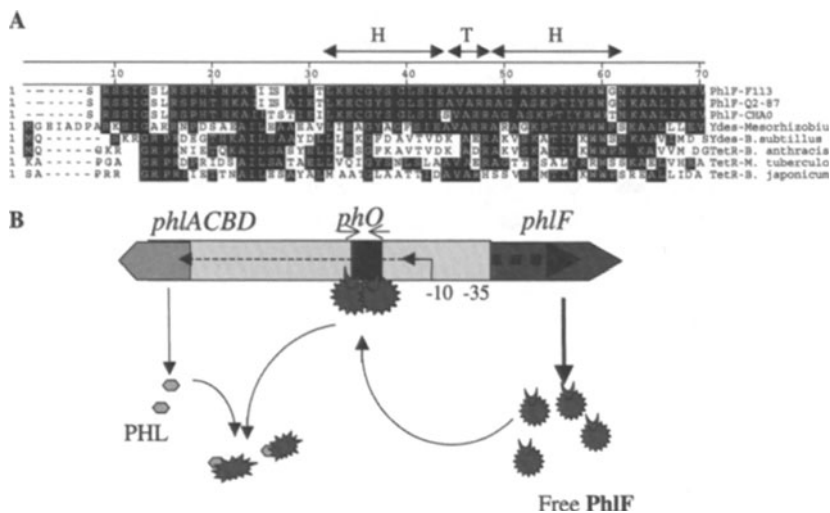


Figure 6. PhlF is a pathway-specific transcriptional repressor. (A) Alignment of PhlF with HTH DNA binding proteins. The N-terminal region of PhlF from three different strains of *P. fluorescens* (F113, CHA0, Q2-87) was aligned with the DNA binding domains of transcriptional regulators that belong to TetR family. YdeS and TetR are widely distributed reference members of this family of transcription regulator. The helix–turn–helix (H–T–H) DNA binding region is indicated. Conserved amino acids are indicated with shading and it is seen that homology is high in the H–T–H region supporting the view that PhlF is a protein in this class of transcriptional regulator. (B) Model showing mode of action of PhlF. PhlF binds to an inverted repeat sequence termed the *phO* operator in the intergenic region between the *phlF* and *phlA* open reading frames. This binding site is downstream of the transcription start site of the *phlA* gene and PhlF binding inhibits transcription, possibly by impairing promoter clearance. PHL acts as an autoinducer by disrupting binding of PhlF to the *phO* operator, thus a positive feedback loop exists.

phlA expression mirrors PHL production and is consistent with the view of PhlF as a transcriptional repressor of PHL biosynthesis. Work *in vivo*⁹⁷ and *in vitro* using purified components² has established molecular details of the mode of repression by PhlF. PhlF, probably acting as a homodimer, recognises and binds to an inverted repeat sequence upstream of the *phlA* open reading frame, termed the *phO* operator. This binding site is downstream of the mapped transcription start site and appears to permit a low level of expression of *phlA*. This is significant, as PHL is now known to act as a positive regulator of its own synthesis by modulating binding of PhlF at the *phlA* promoter (Figure 6B). Other microbial metabolites, such as salicylate, fusaric acid and pyoluteorin, also modulate the activity of *phlF*, though in these cases the metabolites act to strengthen binding of the repressor protein^{2, 97}. Thus production of PHL is subject to control by internal and external metabolites via the pathway specific transcription repressor PhlF.

The influence that environmental factors have on the biosynthesis of secondary metabolites is well illustrated in the case of PHL biosynthesis, which is sensitive to carbon, nitrogen and mineral sources^{26, 30}. Carbon sources such as sucrose, mannitol and fructose promote PHL biosynthesis whereas others such as succinate prevent synthesis. Similarly, certain amino acids (arginine, asparagine and glutamic acid) or nitrates decrease production, whereas other nitrogen sources such as ammonium increase PHL synthesis. It has been demonstrated that, at least in part, this regulation operates *via* modulation of levels of the PhlF protein by regulating transcription of the *phlF* gene. Using *lacZ* transcriptional fusions it was shown that in *P. fluorescens* F113 sucrose increased transcription of *phlACBD* by decreasing that of PhlF. Conversely, on succinate *phlF* expression is increased leading to a decrease in *phlACBD* expression and consequently to a significant reduction in PHL production²⁶. Thus environmental factors and signals can influence production of PHL by modulating both the level and the activity of the PhlF protein.

4.3. Cell Density-Dependent Regulation

In many Gram-negative bacteria, production of secondary metabolites, extracellular enzymes and virulence factors is controlled by a cell–cell signalling system that is generally described as “quorum sensing” or “density-dependent gene regulation”^{36, 37, 95, 100}. The basis of this system is that the bacterium produces autoinducer or signal molecules such as *N*-acyl homoserine lactones (HSLs) that accumulate in the growth medium and trigger expression of target genes when a threshold concentration is reached. The production kinetics of phenazines and PHL make them clear candidates for this type of regulation. It has been established that phenazine biosynthesis is regulated by quorum sensing in *P. aureofaciens*, *P. chlororaphis* and *P. aeruginosa*^{11, 19, 85, 122}. Expression of the phenazine biosynthetic operon in *P. aureofaciens* 30-84 is controlled by PhzI and PhzR, which are members of the LuxI/LuxR family of quorum sensing regulators¹²². *lux* boxes are present upstream of both the *phzI* gene and the *phz* biosynthetic operon, enabling activation of transcription in response to high concentration of the autoinducer C₆-HSL (Figure 7). The central importance of QS for this strain is shown by the data that phenazine production is greatly reduced in a *phzI* mutant and is restored by exogenous production of C₆-HSL by a wild-type strain¹²¹. *P. chlororaphis* PCL1393 also carries *phzI* and *phzR* genes and as is the case with *P. aureofaciens* 30-84, these genes are adjacent to the biosynthetic operon (Figure 2) and control phenazine production in a cell density-dependant manner via C₆-HSL (HHL) production¹⁹.

In *P. fluorescens* 2-79, which produces PCA, the *phzI* and *phzR* genes are also present adjacent to the *phz* biosynthetic operon (Figure 2), and are presumed to also control phenazine biosynthesis. In this bacterium, however,

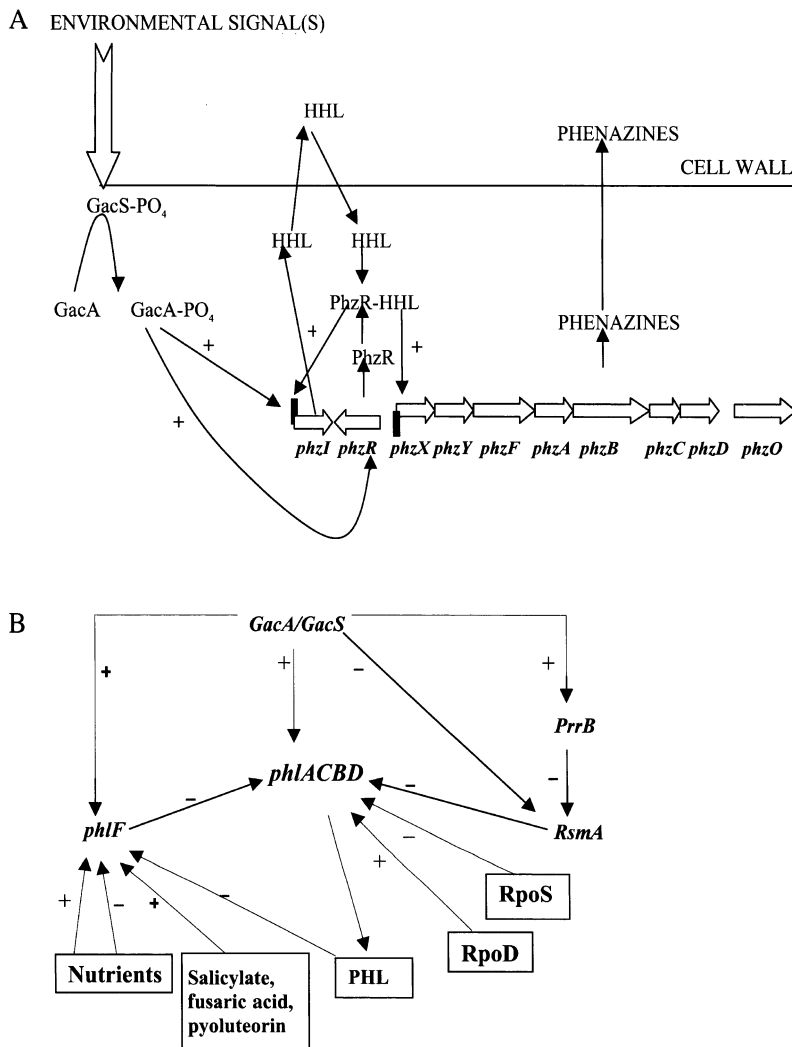


Figure 7. Regulation of production of secondary metabolites. (A) Phenazine regulation. Model for AHL and Gac-mediated control of phenazine production in *P. aureofaciens* 30-84. Potential lux boxes are indicated by solid bars. It is not known whether GacA-mediated effects are direct or indirect, via an intermediary factor (adapted from ref. [83]). (B) Regulation of PHL biosynthesis. A complex regulatory network controls PHL production through regulation of expression of the *phl* biosynthetic genes. The pathway-specific transcriptional repressor PhlF is a key player whose expression and activity is subject to growth phase and environmental regulation. The general transcription factors encoded by *rpoS* and *rpoD* also play a role in the transcriptional regulation. It is also known that post-transcriptional control by the repressor protein RsmA and the regulatory RNA *prfB* also regulate PHL production, possibly by controlling translation of *phlC*. This post-transcriptional system is downstream of the environmental responsive GacS/GacA regulators and thus is part of a global regulatory network.

C₆-HSL is produced at very low levels and other HSLs are likely to have more relevance¹⁵. In *P. aeruginosa* PAO1, the quorum sensing system has been extensively studied and has proved to be very complex, with hierarchical layers of QS regulation^{94, 101}. Pyocyanin biosynthesis is regulated by the rhl QS system¹¹, which itself is regulated by the las QS system⁷⁹, and possibly by the *Pseudomonas* quinolone signal (PQS) system^{38, 78}. Thus, although phenazine biosynthesis is also regulated by QS signalling in *P. aeruginosa* PAO1, the details of this regulation are quite distinct from other *Pseudomonas* bacteria that have been studied.

It has not been established that phloroglucinol production is regulated by density-dependent signals in *P. fluorescens*. Although many strains of *P. fluorescens* produce HSL molecules³², positive correlations between HSL and PHL production have not been made. Indeed, some strains, such as *P. fluorescens* CHAO, which are known to produce PHL appear not to synthesise detectable HSLs. Conversely, *P. fluorescens* F113, which is a strong PHL producer, does synthesise a repertoire of HSL molecules, the functions of which have not yet been determined⁵⁶. Further work will be required to determine whether cell–cell signalling, either by HSLs or by other molecules, plays a role in regulating PHL production.

4.4. Regulation by the Two-Component GacS/GacA System

Two-Component Systems (TCS) regulate bacterial gene expression in response to environmental conditions. These systems consist of a membrane-bound sensor kinase (SK) and a cytoplasmic response regulator (RR). The SK is capable of autophosphorylation in response to an environmental signal(s). This phosphate group is transferred to an RR and the now phosphorylated (activated) RR activates transcription of its target genes. One such TCS present in many plant-associated *Pseudomonads* is the GacS/GacA (Global Antibiotics and Cyanide control) system, which regulates expression of multiple phenotypes including phenazine and PHL production⁴³. In *P. aureofaciens* 30-84, the system controls phenazine biosynthesis via transcriptional regulation of the *phzI* gene (Figure 7A)¹⁶. The level of *phzI* expression in a *gacA* or a *gacS* mutant is less than 8% that of wild type. Expression is restored by complementation of the mutations with wild-type *gacA* or *gacS*. It has been proposed that the Gac system may also control phenazine production in an AHL independent manner since C₆-HSL addition does not restore phenazine production in *gacA* or *gacS* mutants. It is suggested that this second level of phenazine regulation by GacA arises because transcription of the *phz* genes may require a second factor that is under direct or indirect Gac control¹⁶. Quorum Sensing

and the Gac system are also intricately linked in *P. aeruginosa*. The Gac TCS functions upstream of LasR and helps to regulate quorum sensing associated phenotypes in *P. aeruginosa* and thus play a role in pyocyanin production in this bacterium⁹³.

The GacS/GacA regulatory system is required for production of PHL, with mutation of either component completely abolishing PHL biosynthesis⁴³. At one level, transcriptional studies have demonstrated that the GacS/GacA system positively regulates expression of both *phlACBD* and *phlF*²⁶. It has since emerged, however, that a very significant level of control is exerted at the post-transcriptional level. The relevant post-transcriptional control system has been studied in *Escherichia coli*, *Erwinia cartovora* and *Pseudomonas* spp. It comprises a translational repressor protein (CsrA/RsmA) and a regulatory RNA (CsrB/RsmB/PrrB)^{17, 48, 49, 104}. The repressor protein binds to target mRNA molecules at the ribosome binding site, preventing translation and facilitating mRNA degradation. The regulatory RNA can bind and sequester the repressor protein thus allowing translation of those mRNA molecules subject to RsmA repression. Overexpression of the PrrB RNA restored PHL production in GacA/GacS mutants and, conversely, overexpression of *rsmA* mimics the GacA/GacS mutation, abolishing PHL synthesis. It was further demonstrated that wild-type transcription of the *prrB* gene requires the presence of the Gac system¹. These data suggest that RsmA is a downstream regulatory element of the GacA control system in controlling PHL production (Figure 7B). It has not yet been experimentally established which *phl* mRNAs are regulated by RsmA, but sequence analysis suggests that *phlC* may be subject to this translational repression (O'Gara, unpublished).

In *P. aeruginosa*, the link between QS and the Gac system has been expanded to include post-transcriptional regulation as it is now known that the *lasI* mRNA is regulated at the translational level by RsmA⁸⁰. It is clear therefore that the GacS/GacA and *prrB*/RsmA hierarchical system is a major player in the regulation of secondary metabolites in *Pseudomonas* bacteria. Interaction between multiple regulatory circuits presumably allows bacteria to regulate secondary metabolite biosynthesis in a coordinated manner in response to multiple environmental signals.

4.5. Other Regulatory Systems

Other regulators also play direct or indirect roles in controlling the timing and level of secondary metabolite production. The Virulence Factor Regulator (Vfr) protein, which is a homologue of *E. coli* cAMP repressor protein, is implicated in quorum sensing regulation via control of the *las* system^{3, 8}. Alternative use of sigma factors may also be relevant and it has been shown that overexpression of the housekeeping sigma factor *rpoD*, encoding

σ^{70} , or mutation of the *rpoS* gene, encoding σ^s (stationary phase sigma factor), increased PHL production^{96, 117}. In *P. chlororaphis*, PCA production also seems to require an as-yet unidentified factor(s) available in spent growth media. This factor is capable of advancing and increasing PCA biosynthesis and is involved in the positive regulation of the *phzI* gene but is not an HSL¹⁹. Thus in addition to known regulators, it is likely that novel general and specific regulators involved in the control of secondary metabolite production will emerge.

5.1. Distribution of Phenazine Biosynthetic Genes

The phenazine biosynthetic locus is not present in *P. fluorescens* PfO-1 nor is it found in sequenced strains of *P. syringae* pv. tomato strain DC3000 and *P. putida* KT2440, none of which produce phenazines. From analysis of the phenazine biosynthesis loci, the mechanism of phenazine biosynthesis is highly conserved among fluorescent *Pseudomonad* spp. The core biosynthetic operon has been detected via Southern hybridisation in 21 phenazine-producing *Pseudomonads* including strains of *P. aeruginosa*, *P. fluorescens*, *P. chlororaphis* and *P. aureofaciens*⁶⁴. This operon was not detected in seven phenazine-producing isolates of *Burkholderia cepacia*, *Burkholderia phenazinium* and *Brevibacterium iodinum*. This indicates that the phenazine core pathway is highly conserved in fluorescent *Pseudomonads* but differs from that in other phenazine-producing bacteria, and that phenazine production probably predates strain differentiation within the fluorescent *Pseudomonads*. This implies that the phenazine biosynthetic locus has been lost from some lineages of *Pseudomonas* during evolution, whereas others such as the *P. chlororaphis* lineage have maintained the locus. No evidence has been put forward to support an alternative possibility of gene acquisition. Detailed analysis of the molecular evolution and distribution of the genes encoding enzymes that modify PCA to other phenazines have not been carried out, though it was shown by Southern hybridisation that the *phzO* gene is found almost exclusively in *P. aureofaciens* isolates²⁴.

5.2. Genotypic Diversity among PHL-Producing *Pseudomonads*

The positive correlation between disease suppressive soils and the presence of high numbers of phloroglucinol-producing *Pseudomonads* is well established (cf. introduction). It is less well known, however, what role plants play in the selection of specific PHL-producing strains, or how genetic diversity between strains influences PHL biosynthesis. A number of studies have explored diversity among PHL-producing *Pseudomonas* bacteria to try to

address these issues. Phylogenetic groupings have been deduced from analysis of 16S rRNA sequences using ARDRA, and from whole genome analysis using RAPD, ERIC and BOX primer sets. Although interpretation of the collective data sets are hindered by the fact that not all workers have used identical methods and primers sets, the general findings are consistent between studies. ARDRA analysis identifies 3–4 phylogenetic groups, with RAPD methods identifying between 17 and 39 further subgroups within these, depending on the primer sets and strains chosen^{55, 64, 68, 81, 82, 91, 92, 115}. Since it is known that not all strains have equivalent biocontrol ability, considerable effort has been devoted to analysing diversity within the *phl* biosynthetic locus of these strains. Based on Southern blotting of a limited number of strains, it was suggested that all PHL-producing *Pseudomonas* carry the *phlA-D* biosynthetic genes⁵⁴, and the demonstration that *phlD* sequences can be amplified by PCR from all PHL-producing strains supports this conclusion¹¹⁵. RFLP analysis by several groups indicated that there is a high level of diversity in the *phlD* gene but the significance of this for PHL production remains unclear^{55, 64, 68, 81, 82, 91, 92, 115}. Attempts have been made to correlate particular *phlD* genotypes with levels of PHL production in vitro and some associations do occur^{81, 91}. These, however, tend to be growth medium-specific, and the relationship between in vitro production and levels in the rhizosphere is unknown.

Phloroglucinol production is not universal among *Pseudomonads*, with some studies showing the percentage of producers in soils being as low as 1–2% of the *Pseudomonas* population. The percentage of PHL producers is considerably higher in plant rhizospheres and in soil that has been subjected to extensive monoculture with some plant species^{81, 90}, indicating that there is a strong selective pressure exerted by plants to encourage the proliferation of PHL-producers. This leads to the question as to whether specific strain genotypes or specific *phlD* genotypes are being selected? Most studies have reported tight correlations between strain phylogeny (ARDRA and RAPD groups) and phylogeny inferred from *phlD* sequences thus precluding a definitive answer to this question. Some clear findings have emerged, however. In soils that have been exposed to wheat monoculture, a single *Pseudomonas* genotype predominated in the population⁶⁴, and certain genotypes were selected by Pea cultivars growing in *Fusarium*-suppressive soil⁵⁵. These results, and subsequent analysis of representative strains^{66, 89}, show that plants do select for specific *Pseudomonas* genotypes, though whether this relates to PHL-production or to other traits has not been established. Counterbalancing these data, another study found that single plants maintained *Pseudomonas* populations carrying distinct *phlD* alleles, indicating that selection need not be completely exclusive⁹². Some of the apparent discrepancies between the studies may be accounted for by differences between plant types (esp. monocot/dicot) and also by different soil histories (esp. monoculture vs mixed culture).

The variable distribution of the *phl* biosynthetic operon among *Pseudomonas* has led to speculation on the evolutionary history of these genes. Sequence analysis of *phlD* established that it encodes a type III polyketide synthase that is unusual in prokaryotes⁷. Genes in this family are found in some Gram-positive bacteria and are common in plants where they encode STS and CHS enzymes. These plant enzymes are branchpoints for the biosynthesis of plant secondary metabolites that play roles in defence and signalling (e.g., stilbenes and flavonoids), thus the unexpected presence of homologues in plant-associated *Pseudomonads* is intriguing. It has been speculated that the *Pseudomonas phlD* gene was acquired from plants by horizontal gene transfer, though conflicting or incomplete data leave this an open question. The correlations between whole genome and *phlD* phylogenies^{55, 91} would argue against recent horizontal transfer, and separate clustering of type III PKS genes from plant, *Pseudomonas* and Gram-positive bacteria has been interpreted to suggest independent convergent evolution⁹¹. Conversely, conservation of key residues between *Pseudomonas* and plant enzymes⁹¹, and some clear anomalies in the phylogenetic associations^{55, 81} would support the idea of horizontal acquisition. Other observations such as the absence of the *phl* operon in *P. fluorescens* Pf01, which is closely related to producing strain *P. fluorescens* F113, suggest that further analysis will be required to provide a definitive answer to this question. More genotypic analysis on non-producing strains, and clarification of *Pseudomonas* taxonomy and evolutionary relationships, should contribute to this question. Very little attention has been focused on the ancestry of other genes in the *phl* operon. The closest homologues to the *phlA*, *phlC* and *phlB* genes, which are reported to be co-transcribed with *phlD*, are found in archaea⁷. Homologues of *phlE* and *phlF* are found in other Eubacteria. Based on the limited data available, a scenario emerges whereby genes came together from diverse origins to form the genetic locus responsible for synthesis of PHL. A more complete understanding of the biological effects of PHL may cast light on the selection pressures that may have facilitated this process.

6. BIOLOGICAL ROLE OF SECONDARY METABOLITES IN PSEUDOMONADS

The rationale for much of the research on biosynthesis of secondary metabolites by *Pseudomonas* has been the potent antimicrobial activity of metabolites such as phenazines and phloroglucinol. The general view of these metabolites is that their biological role is to confer an advantage on the producing strains over non-producing bacteria in competitive ecological niches. This would be analogous to antibiotic production by *Streptomyces* or bacteriocin production by lactic acid bacteria. Competitor exclusion may well

be the primary function of these secondary metabolites, and maximal synthesis in late logarithmic/stationary phase would be consistent with this role. As diverse roles for other bacterial metabolites emerge, however, it may be appropriate to also consider the possibility of alternative biological roles for some of these antimicrobial metabolites. The kinetics of phloroglucinol production are particularly intriguing. Synthesis is tightly controlled, primarily at the transcriptional level, to allow a rapid burst of synthesis towards the end of logarithmic growth. Rather than remaining at a high level, however, PHL concentrations rapidly fall early in stationary phase, with PHL either degraded or assimilated into a larger molecule (Figure 4B). This pattern of production is more akin to that seen with autoinducer signals such as HSLs, than that which might be expected from an antimicrobial metabolite. Indeed, there is precedent for signal molecules being classified as antimicrobials; for example, the discovery that the “*small*” bacteriocin from *Rhizobium* is in fact a HSL that controls hierarchal gene expression in that bacterium¹¹⁹. The identification of the PQS may also be salient, as quinolones have been studied for their antimicrobial properties. Further consideration of possible alternative biological roles for PHL may also contribute to understanding how and why plants appear to select for PHL-producing *Pseudomonas* bacteria. In this regard, two observations stand out. First, the plant homologues, and possible evolutionary ancestors, of *phlD* encode enzymes involved in synthesis of both antimicrobial and signal molecules; and second, plants have also been reported to select for *Pseudomonas* strains with the capacity to produce HSL signal molecules³². This second observation suggests that a benefit to the plant may accrue by encouraging the growth of signal-producing *Pseudomonas*, though how this is sensed or mediated is unknown. Some question marks also remain over the importance of phenazine biosynthesis. Although expression of the phenazine-1-carboxylic acid operon in a strain of *P. putida* did not alter ecological fitness⁴⁰, studies with both *P. aurofaciens* 30-84 and *P. fluorescens* 2-79 reported reduced survival characteristics for non-producing mutants of these strains⁶⁷. More in-depth studies of all these phenomena are certainly warranted.

7.1. Exploiting Knowledge of Secondary Metabolite Biosynthesis to Generate Improved Biocontrol Strains

Despite the natural ability of *Pseudomonads* to suppress plant pathogens, there has been limited success in developing effective commercial biocontrol products based on these strains. From a scientific perspective, this is in part due to variable performance of biocontrol strains due to unpredictable levels on

secondary metabolite production, narrow ranges of protection or poor colonisation under the prevailing environmental conditions. Knowledge of the synthesis and regulation of secondary metabolites affords an opportunity to apply rationale design strategies to generate strains with improved biocontrol potential.

7.2. Genetic Enhancement of Biocontrol Strains

The clustered organisation of the biosynthetic genes involved in the biosynthesis of secondary metabolites, such as DAPG, phenazines, pyoluteorin and pyrrolnitrin^{7, 44, 65, 76}, facilitates the introduction of novel biosynthetic capability to create strains with enhanced biocontrol traits. Re-introduction of extra copies of the *phl* biosynthetic locus into PHL-producing strains *P. fluorescens* CHAO and F113 created strains that produced higher levels of PHL and had improved biocontrol ability^{27, 63}. It has also been possible to introduce biosynthetic operons into non-producing strains to create new biocontrol strains. Transfer of the *phl* operon into the non-biocontrol strain *P. fluorescens* M114 resulted in a strain with biocontrol activity against the phytopathogenic oomycete *Pythium ultimum*³⁴, and introduction of the core phenazine-1-carboxylic acid biosynthetic operon into the (non-producing) biocontrol strains *P. putida* WCS358 and *P. fluorescens* SBW25 enhanced the biocontrol abilities of those strains^{5, 40, 105}. Knowing the precise biosynthetic pathways also allows precise metabolic engineering of the secondary metabolite profile of a strain. Biocontrol strains *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 contain the core PCA biosynthetic operon and synthesise PCA. Introduction of the *phzH* gene from *P. chlororaphis* into these strains enables them to produce PCN and expanded the range of phytopathogens that could be controlled by these bacteria²¹. Despite these successes, there are still limitations with this approach. As discussed above, biosynthesis of secondary metabolites is tightly controlled by multiple regulatory systems, including some operating at the post-transcriptional level. Thus, it can be difficult to construct strains that produce sufficient levels of the metabolite at the appropriate time to control disease. Increasing the copy number of the *phl* operon in *P. fluorescens* F113 did increase the level of PHL produced by this strain but did not lead to earlier or more sustained production²⁷, and disruption of the transcriptional repressor *phlF* led to advanced production but to the same level as the wild-type strain. A reasonable conclusion from all these studies is that genetic manipulation certainly has the potential to generate improved strains, but further knowledge of all the regulatory processes governing production is required to allow directed approaches to overcome these specific controls without impairing ecological fitness in the bacterium.

7.3. Manipulation of Environmental Signals to Enhance Biocontrol

Knowledge of how the regulatory systems that control secondary metabolite production operate creates opportunities to manipulate these pathways using environmental cues and signals. This can be considered a complementary approach to reprogramming pathways through genetic engineering as described in Section 7.2. With regard to synthesis of phloroglucinol, a number of molecules that enhance or reduce synthesis have been identified. The signals studied to date work by modifying the levels or activity of the transcriptional repressor PhlF. Thus, adding succinate to cells leads to high levels of the PhlF protein and consequent low levels of PHL, with other carbon and nitrogen sources having opposite effects (O’Gara, unpublished). Other signals such as PHL itself, salicylic acid and fusaric acid modulate the interaction between the PhlF and its target site at the *phlA* promoter, thereby altering PHL levels^{2,75,97}. Adding amendments containing modifiers can alter both PHL production and the biocontrol abilities of strains of *P. fluorescens* (Mark and O’Gara, unpublished)³⁰. As further information on how regulatory pathways are controlled becomes available, it should be possible to design strain-specific supplements that will manipulate timing and levels of secondary metabolite production.

8. CONCLUSIONS

A large body of data contributing to our understanding of secondary metabolite production in *Pseudomonas* bacteria has been accumulated. Nonetheless, it is quite clear that there are still many unanswered questions and many details that need to be completed. The limitations of our knowledge are exemplified by the limited success in generating strains of *P. fluorescens* that dramatically overproduce PHL. Obviously, construction of overproducing strains is complicated by the fact that several of the regulatory circuits also control other processes in the cell. A knowledge gap also exists between the performance of strains in the laboratory and *in situ* in the rhizosphere. Advances in *in situ* technologies such as lux or gfp reporter systems now provide the tools to thoroughly characterise gene expression *in situ* and should deliver answers to questions such as whether *in vitro* and *in situ* levels are comparable, and how environmental conditions influence expression of regulatory and biosynthetic genes. This may go some way to addressing why such variability in field performance has been observed in some studies.

In developing future microbial biocontrol products, it may be necessary to consider integrating some of the strategies outlined in Section 7 with the

development of compatible and complementary microbial consortia. Combining biocontrol strains that have different targets/mode of action/kinetics of production may be one route forward. If this is integrated with knowledge on genetic pathway and interspecies signalling, it will create exciting opportunities in this field. The potential of this approach was shown by the finding of synergistic interaction between *P. fluorescens* lipodepsipeptides and cell wall degrading enzymes produced by *Trichoderma*^{35, 120}.

Further consideration also needs to be given to the regulatory and biosafety issues regarding microbial biocontrol strains. The general presumption is that since these are “natural” products, microbial inoculants are intrinsically safer and more environmentally friendly than agrochemicals. Although there are grounds for believing this to be so, in many cases there has not been clear demonstrations that this is, in fact, the case. The issues surrounding GM strains are well documented, but there are certain similarities regarding biocontrol strains and their metabolites. This has received attention from regulatory authorities and it is likely that there will be an increased onus in the future to demonstrate that these strains are safe to humans and do not constitute an appreciable risk to non-target organisms (e.g. see ref. [69], [114]). This will entail a more detailed understanding of the biology of secondary metabolites and their mode of action and effects on target and non-target organisms than we currently possess. It is clear therefore, that from several perspectives there is a strong scientific rationale for continuing studies on secondary metabolite production in *Pseudomonas*.

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REFERENCES

1. Aarons, S., Abbas, A., Adams, C., Fenton, A., and O’Gara, F., 2000, A regulatory RNA (PrrB RNA) modulates expression of secondary metabolite genes in *Pseudomonas fluorescens* F113. *J. Bacteriol.*, 182:3913–3919.
2. Abbas, A., Morrissey, J.P., Marquez, P.C., Sheehan, M.M., Delany, I.R., and O’Gara, F., 2002, Characterization of interactions between the transcriptional repressor PhlF and its binding site at the *phlA* promoter in *Pseudomonas fluorescens* F113. *J. Bacteriol.*, 184:3008–3016.

3. Albus, A.M., Pesci, E.C., Runyen-Janecky, L.J., West, S.E., and Iglewski, B.H., 1997, Vfr controls quorum sensing in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 179:3928–3935.
4. Audenaert, K., Pattery, T., Cornelis, P., and Hofte, M., 2002, Induction of systemic resistance to *Botrytis cinerea* in tomato by *Pseudomonas aeruginosa* 7NSK2: Role of salicylic acid, pyochelin, and pyocyanin. *Mol. Plant Microbe Interact.*, 15:1147–1156.
5. Bakker, P.A., Glandorf, D.C., Viebahn, M., Ouwens, T.W., Smit, E., Leeftang, P., Wernars, K., Thomashow, L.S., Thomas-Oates, J.E., and van Loon, L.C., 2002, Effects of *Pseudomonas putida* modified to produce phenazine-1-carboxylic acid and 2,4-diacetylphloroglucinol on the microflora of field grown wheat. *Antonie Van Leeuwenhoek*, 81:617–624.
6. Bangera, M.G. and Thomashow, L.S., 1996, Characterization of a genomic locus required for synthesis of the antibiotic 2,4-diacetylphloroglucinol by the biological control agent *Pseudomonas fluorescens* Q2-87. *Mol. Plant Microbe Interact.*, 9:83–90.
7. Bangera, M.G. and Thomashow, L.S., 1999, Identification and characterization of a gene cluster for synthesis of the polyketide antibiotic 2,4-diacetylphloroglucinol from *Pseudomonas fluorescens* Q2-87. *J. Bacteriol.*, 181:3155–3163.
8. Beatson, S.A., Whitchurch, C.B., Sargent, J.L., Levesque, R.C., and Mattick, J.S., 2002, Differential regulation of twitching motility and elastase production by Vfr in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 184:3605–3613.
9. Bloemberg, G.V. and Lugtenberg, B.J., 2001, Molecular basis of plant growth promotion and biocontrol by rhizobacteria. *Curr. Opin. Plant Biol.*, 4:343–350.
10. Bonsall, R.F., Weller, D.M., and Thomashow, L.S., 1997, Quantification of 2,4-diacetylphloroglucinol produced by fluorescent *Pseudomonas* spp. In vitro and in the rhizosphere of wheat. *Appl. Environ. Microbiol.*, 63:951–955.
11. Brint, J.M. and Ohman, D.E., 1995, Synthesis of multiple exoproducts in *Pseudomonas aeruginosa* is under the control of RhlR-RhlI, another set of regulators in strain PAO1 with homology to the autoinducer-responsive LuxR-LuxI family. *J. Bacteriol.*, 177:7155–7163.
12. Budzikiewicz, H., 1993, Secondary metabolites from fluorescent pseudomonads. *FEMS Microbiol. Rev.*, 10:209–228.
13. Campbell, M.M., Sainsbury, M., and Searle, P.A., 1993, The biosynthesis and synthesis of shikimic acid, chorismic acid and related compounds. *Synthesis*, 2:179–193.
14. Carroll, H., Moënné-Loccoz, Y., Dowling, D.N., and O’Gara, F., 1995, Mutational disruption of the biosynthesis genes coding for the antifungal metabolite 2,4-diacetylphloroglucinol does not influence the ecological fitness of *Pseudomonas fluorescens* F113 in the rhizosphere of sugarbeets. *Appl. Environ. Microbiol.*, 61:3002–3007.
15. Cha, C., Gao, P., Chen, Y.C., Shaw, P.D., and Farrand, S.K., 1998, Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. *Mol. Plant Microbe Interact.*, 11:1119–1129.
16. Chancey, S.T., Wood, D.W., and Pierson, L.S., III, 1999, Two-component transcriptional regulation of N-acyl-homoserine lactone production in *Pseudomonas aureofaciens*. *Appl. Environ. Microbiol.*, 65:2294–2299.
17. Chatterjee, A., Cui, Y., and Chatterjee, A.K., 2002, RsmA and the quorum-sensing signal, N-[3-oxohexanoyl]-L-homoserine lactone, control the levels of rsmB RNA in *Erwinia carotovora* subsp. *carotovora* by affecting its stability. *J. Bacteriol.*, 184:4089–95.
18. Chin-A-Woeng, T.F., Bloemberg, G.V., Mulders, I.H., Dekkers, L.C., and Lugtenberg, B.J., 2000, Root colonization by phenazine-1-carboxamide-producing bacterium *Pseudomonas chlororaphis* PCL1391 is essential for biocontrol of tomato foot and root rot. *Mol. Plant Microbe Interact.*, 13:1340–1345.
19. Chin-A-Woeng, T.F., van den Broek, D., de Voer, G., van der Drift, K.M., Tuinman, S., Thomas-Oates, J.E., Lugtenberg, B.J., and Bloemberg, G.V., 2001, Phenazine-1-carboxamide production in the biocontrol strain *Pseudomonas chlororaphis* PCL1391 is regulated by multiple factors secreted into the growth medium. *Mol. Plant Microbe Interact.*, 14:969–979.

20. Chin-A-Woeng, T.F.C., Bloemberg, G.V.A., van der Bij, J., van der Drift, K.M.G.M., Schripsema, J., Kroon, B., Scheffer, R.J., Keel, C., Bakker, P.A.H.M., Tichy, H.-V., de Bruijn, F.J., Thomas-Oates, J.E., and Lugtenberg, B.J.J., 1998, Biocontrol by phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* PCL1391 of tomato root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersic*. *Mol. Plant-Microbe Interact.*, 11:1069–1077.
21. Chin-A-Woeng, T.F.C., Thomas-Oates, J.E., Lugtenberg, B.J., and Bloemberg, G.V., 2001, Introduction of the *phzH* gene of *Pseudomonas chlororaphis* PCL1391 extends the range of biocontrol ability of phenazine-1-carboxylic acid-producing *Pseudomonas* spp. strains. *Mol. Plant Microbe Interact.*, 14:1006–1015.
22. Cornelis, P. and Matthijs, S., 2002, Diversity of siderophore-mediated iron uptake systems in fluorescent pseudomonads: Not only pyoverdines. *Environ. Microbiol.*, 4:787–798.
23. de Lorenzo, V., 2001, The potential for genetically modified bacteria to break down toxic pollutants in the environment. *EMBO reports*, 2:357–359.
24. Delaney, S.M., Mavrodi, D.V., Bonsall, R.F., and Thomashow, L.S., 2001, *phzO*, a gene for biosynthesis of 2-hydroxylated phenazine compounds in *Pseudomonas aureofaciens* 30–84. *J. Bacteriol.*, 183:318–327.
25. Delany, I., Sheehan, M.M., Fenton, A., Bardin, S., Aarons, S., and O’Gara, F., 2000, Regulation of production of the antifungal metabolite 2,4-diacetylphloroglucinol in *Pseudomonas fluorescens* F113: Genetic analysis of PhlF as a transcriptional repressor. *Microbiology*, 146:537–543.
26. Delany, I.R., 1999, PhD thesis. University College Cork.
27. Delany, I.R., Walsh, U.F., Ross, I., Fenton, A.M., Corkery, D.M., and O’Gara, F., 2001, Enhancing the biocontrol efficacy of *Pseudomonas fluorescens* F113 by altering the regulation and production of 2,4-diacetylphloroglucinol. *Plant Soil*, 232:195–205.
28. Denning, G.M., Railsback, M.A., Rasmussen, G.T., Cox, C.D., and Britigan, B.E., 1998, *Pseudomonas* pyocyanine alters calcium signaling in human airway epithelial cells. *Am. J. Physiol.*, 274:L893–900.
29. Denning, G.M., Wollenweber, L.A., Railsback, M.A., Cox, C.D., Stoll, L.L., and Britigan, B.E., 1998, *Pseudomonas* pyocyanin increases interleukin-8 expression by human airway epithelial cells. *Infect. Immun.*, 66:5777–5784.
30. Duffy, B.K. and Defago, G., 1999, Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. *Appl. Environ. Microbiol.*, 65:2429–2438.
31. Earhart, C.F., 1996, Uptake and metabolism of iron and molybdenum. pp. 1075–1090. In F.C. Neidhardt, R. Curtiss, III, J.L. Ingraham, E.C.C. Lin, K.B. Low, B. Magasanik, W.S. Reznikoff, M. Riley, S.M., and H.E. Umbarger (eds), *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, pp. 1075–1090. ASM Press, Washington, D.C.
32. Elasri, M., Delorme, S., Lemanceau, P., Stewart, G., Laue, B., Glickmann, E., Oger, P.M., and Dessaux, Y., 2001, Acyl-homoserine lactone production is more common among plant-associated *Pseudomonas* spp. than among soilborne *Pseudomonas* spp. *Appl. Environ. Microbiol.*, 67:1198–1209.
33. Fakhouri, W., Walker, F., Vogler, B., Armbruster, W., and Buchenauer, H., 2001, Isolation and identification of N-mercapto-4-formylcarbostyryl, an antibiotic produced by *Pseudomonas fluorescens*. *Phytochemistry*, 58:1297–1303.
34. Fenton, A.M., Stephens, P.M., Crowley, J., O’Callaghan, M., and O’Gara, F., 1992, Exploitation of gene(s) involved in 2,4-diacetylphloroglucinol biosynthesis to confer a new biocontrol capability to a *Pseudomonas* strain. *Appl. Environ. Microbiol.*, 58:3873–3878.
35. Fogliano, V., Ballio, A., Gallo, M., Woo, S., Scala, F., and Lorito, M., 2002, *Pseudomonas* lipodepsipeptides and fungal cell wall-degrading enzymes act synergistically in biological control. *Mol. Plant Microbe Interact.*, 15:323–333.

36. Fuqua, C., Winans, S.C., and Greenberg, E.P., 1996, Census and consensus in bacterial ecosystems: The LuxR-LuxI family of quorum-sensing transcriptional regulators. *Annu. Rev. Microbiol.*, 50:727–751.
37. Fuqua, W.C., Winans, S.C., and Greenberg, E.P., 1994, Quorum sensing in bacteria: The LuxR-LuxI family of cell density-responsive transcriptional regulators. *J. Bacteriol.*, 176:269–275.
38. Gallagher, L.A., McKnight, S.L., Kuznetsova, M.S., Pesci, E.C., and Manoil, C., 2002, Functions required for extracellular quinolone signaling by *Pseudomonas aeruginosa*. *J. Bacteriol.*, 184:6472–6480.
39. Gamard, P., Sauriol, F., Benhamou, N., Belanger, R.R., and Paulitz, T.C., 1997, Novel butyrolactones with antifungal activity produced by *Pseudomonas aureofaciens* strain 63–28. *J. Antibiot. (Tokyo)*, 50:742–749.
40. Glandorf, D.C., Verheggen, P., Jansen, T., Jorritsma, J.W., Smit, E., Leeftang, P., Wernars, K., Thomashow, L.S., Laureijs, E., Thomas-Oates, J.E., Bakker, P.A., and van Loon, L.C., 2001, Effect of genetically modified *Pseudomonas putida* WCS358r on the fungal rhizosphere microflora of field-grown wheat. *Appl. Environ. Microbiol.*, 67:3371–3378.
41. Gurusiddaiah, S., Weller, D.M., Sarkar, A., and Cook, R.J., 1986, Characterization of an antibiotic produced by a strain of *Pseudomonas fluorescens* inhibitory to *Gaeumannomyces graminis* var. *tritici* and *Pythium* spp. *Antimicrob. Agents Chemother.*, 29:488–495.
42. Gutterson, N.I., Layton, T.J., Ziegler, J.S., and Warren, G.J., 1986, Molecular cloning of genetic determinants for inhibition of fungal growth by a fluorescent pseudomonad. *J. Bacteriol.*, 165:696–703.
43. Haas, D., Blumer, C., and Keel, C., 2000, Biocontrol ability of fluorescent pseudomonads genetically dissected: Importance of positive feedback regulation. *Curr. Opin. Biotechnol.*, 11:290–297.
44. Hammer, P.E., Burd, W., Hill, D.S., Ligon, J.M., and van Pee, K., 1999, Conservation of the pyrrolnitrin biosynthetic gene cluster among six pyrrolnitrin-producing strains. *FEMS Microbiol. Lett.*, 180:39–44.
45. Hassan, H.M. and Fridovich, I., 1980, Mechanism of the antibiotic action pyocyanine. *J. Bacteriol.*, 141:156–163.
46. Hassett, D.J., Charniga, L., Bean, K., Ohman, D.E., and Cohen, M.S., 1992, Response of *Pseudomonas aeruginosa* to pyocyanin: Mechanisms of resistance, antioxidant defenses, and demonstration of a manganese-cofactored superoxide dismutase. *Infect Immun.*, 60:328–336.
47. Hassett, D.J., Schweizer, H.P., and Ohman, D.E., 1995, *Pseudomonas aeruginosa* *sodA* and *sodB* mutants defective in manganese- and iron-cofactored superoxide dismutase activity demonstrate the importance of the iron-cofactored form in aerobic metabolism. *J. Bacteriol.*, 177:6330–6337.
48. Heeb, S., Blumer, C., and Haas, D., 2002, Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct formation in *Pseudomonas fluorescens* CHA0. *J. Bacteriol.*, 184:1046–1056.
49. Heeb, S. and Haas, D., 2001, Regulatory roles of the GacS/GacA two-component system in plant-associated and other gram-negative bacteria. *Mol. Plant Microbe Interact.*, 14:1351–1363.
50. Hollstein, U. and Marshall, L.G., 1972, Biosynthesis of phenazines. *J. Org. Chem.*, 37:3510–3514.
51. Hollstein, U. and McCamey, D.A., 1973, Biosynthesis of phenazines. II. Incorporation of (6-14C)-D-shikimic acid into phenazine-1-carboxylic acid and iodinin. *J. Org. Chem.*, 38:3415–3417.
52. Howell, C.D. and Stipanovic, R.D., 1980, Suppression of *Pythium ultimum*-induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic, pyoluteorin. *Phytopathology*, 70:712–715.

53. Hussain, A.S., Bozinovski, J., Maurice, D.H., McLaughlin, B.E., Marks, G.S., Brien, J.F., and Nakatsu, K., 1997, Inhibition of the action of nitric oxide prodrugs by pyocyanin: Mechanistic studies. *Can. J. Physiol. Pharmacol.*, 75:398–406.
54. Keel, C., Weller, D.M., Natsch, A., Defago, G., Cook, R.J., and Thomashow, L.S., 1996, Conservation of the 2,4-diacetylphloroglucinol biosynthesis locus among fluorescent *Pseudomonas* strains from diverse geographic locations. *Appl. Environ. Microbiol.*, 62:552–563.
55. Landa, B.B., Mavrodi, O.V., Raaijmakers, J.M., B.B. McSpadden Gardener, Thomashow, L.S., and Weller, D.M., 2002, Differential ability of genotypes of 2,4-diacetylphloroglucinol-producing *Pseudomonas fluorescens* strains to colonize the roots of pea plants. *Appl. Environ. Microbiol.*, 68:3226–3237.
56. Laue, B.E., Jiang, Y., Chhabra, S.R., Jacob, S., Stewart, G.S., Hardman, A., Downie, J.A., O’Gara, F., and Williams, P., 2000, The biocontrol strain *Pseudomonas fluorescens* F113 produces the *Rhizobium* small bacteriocin, N-(3-hydroxy-7-cis-tetradecenoyl)homoserine lactone, via HdtS, a putative novel N-acylhomoserine lactone synthase. *Microbiology*, 146:2469–2480.
57. Lauredo, I.T., Sabater, J.R., Ahmed, A., Botvinnikova, Y., and Abraham, W.M., 1998, Mechanism of pyocyanin- and 1-hydroxyphenazine-induced lung neutrophilia in sheep airways. *J. Appl. Physiol.*, 85:2298–2304.
58. Laville, J., Voisard, C., Keel, C., Maurhofer, M., Defago, G., and Haas, D., 1992, Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. *Proc. Natl. Acad. Sci. USA* 89:1562–1566.
59. Lee, J.Y., Moon, S.S., and Hwang, B.K., 2003, Isolation and antifungal and antioomycete activities of aerugine produced by *Pseudomonas fluorescens* strain MM-B16. *Appl. Environ. Microbiol.*, 69:2023–2031.
60. Leisinger, T. and Margraff, R., 1979, Secondary metabolites of the fluorescent pseudomonads. *Microbiol. Rev.*, 43:422–442.
61. Longley, R.P., Halliwell, J.E., Campbell, J.J., and Ingledew, W.M., 1972, The branchpoint of pyocyanine biosynthesis. *Can. J. Microbiol.*, 18:1357–1363.
62. Massiere, F. and Badet-Denisot, M.A., 1998, The mechanism of glutamine-dependent amidotransferases. *Cell Mol. Life Sci.*, 54:205–222.
63. Mauerhofer, M., Keel, C., Haas, D., and Defago, G., 1995, Influence of plant species on disease suppression by *Pseudomonas fluorescens* strain CHA0 with enhanced antibiotic production. *Plant Pathol.*, 44:40–50.
64. Mavrodi, D.V., Bonsall, R.F., Delaney, S.M., Soule, M.J., Phillips, G., and Thomashow, L.S., 2001, Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PAO1. *J. Bacteriol.*, 183:6454–6465.
65. Mavrodi, D.V., Ksenzenko, V.N., Bonsall, R.F., Cook, R.J., Boronin, A.M., and Thomashow, L.S., 1998, A seven-gene locus for synthesis of phenazine-1-carboxylic acid by *Pseudomonas fluorescens* 2-79. *J. Bacteriol.*, 180:2541–2548.
66. Mavrodi, D.V., Mavrodi, O.V., McSpadden-Gardener, B.B., Landa, B.B., Weller, D.M., and Thomashow, L.S., 2002, Identification of differences in genome content among *phlD*-positive *Pseudomonas fluorescens* strains by using PCR-based subtractive hybridization. *Appl. Environ. Microbiol.*, 68:5170–5176.
67. Mazzola, M., Cook, R.J., Thomashow, L.S., Weller, D.M., and Pierson, L.S., 3rd, 1992, Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent pseudomonads in soil habitats. *Appl. Environ. Microbiol.*, 58:2616–2624.
68. McSpadden Gardener, B.B., Schroeder, K.L., Kalloger, S.E., Raaijmakers, J.M., Thomashow, L.S., and Weller, D.M., 2000, Genotypic and phenotypic diversity of *phlD*-containing *Pseudomonas* strains isolated from the rhizosphere of wheat. *Appl. Environ. Microbiol.*, 66:1939–1946.

69. Morrissey, J.P., Walsh, U.F., O'Donnell, A., Moenne-Loccoz, Y., and O'Gara, F., 2002, Exploitation of genetically modified inoculants for industrial ecology applications. *Antonie Van Leeuwenhoek*, 81:599–606.
70. Neilands, J.B., 1982, Microbial envelope proteins related to iron. *Annu. Rev. Microbiol.*, 36:285–309.
71. Nelson, K.E., 2002, The complete genome sequence of *Pseudomonas putida* KT2440 is finally available. *Environ. Microbiol.*, 4:777–778.
72. Nelson, K.E., Weinel, C., Paulsen, I.T., Dodson, R.J., Hilbert, H., Martins dos Santos, V.A., Fouts, D.E., Gill, S.R., Pop, M., Holmes, M., Brinkac, L., Beanan, M., DeBoy, R.T., Daugherty, S., Kolonay, J., Madupu, R., Nelson, W., White, O., Peterson, J., Khouri, H., Hance, I., Chris, Lee, P., Holtzapple, E., Scanlan, D., Tran, K., Moazzez, A., Utterback, T., Rizzo, M., Lee, K., Kosack, D., Moestl, D., Wedler, H., Lauber, J., Stjepandic, D., Hoheisel, J., Straetz, M., Heim, S., Kiewitz, C., Eisen, J., Timmis, K.N., Dusterhoft, A., Tummli, B., and Fraser, C.M., 2002, Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ. Microbiol.*, 4:799–808.
73. Nielsen, T.H., Christophersen, C., Anthoni, U., and Sorensen, J., 1999, Viscosinamide, a new cyclic depsipeptide with surfactant and antifungal properties produced by *Pseudomonas fluorescens* DR54. *J. Appl. Microbiol.*, 87:80–90.
74. Nielsen, T.H., Thrane, C., Christophersen, C., Anthoni, U., and Sorensen, J., 2000, Structure, production characteristics and fungal antagonism of tensin-a new antifungal cyclic lipopeptide from *Pseudomonas fluorescens* strain 96.578. *J. Appl. Microbiol.*, 89:992–1001.
75. Notz, R., Maurhofer, M., Dubach, H., Haas, D., and Defago, G., 2002, Fusaric acid-producing strains of *Fusarium oxysporum* alter 2,4-diacetylphloroglucinol biosynthetic gene expression in *Pseudomonas fluorescens* CHA0 in vitro and in the rhizosphere of wheat. *Appl. Environ. Microbiol.*, 68:2229–2235.
76. Nowak-Thompson, B., Chaney, N., Wing, J.S., Gould, S.J., and Loper, J.E., 1999, Characterization of the pyoluteorin biosynthetic gene cluster of *Pseudomonas fluorescens* Pf-5. *J. Bacteriol.*, 181:2166–2174.
77. Pedras, M.S., Ismail, N., Quail, J.W., and Boyetchko, S.M., 2003, Structure, chemistry, and biological activity of pseudophomins A and B, new cyclic lipodepsipeptides isolated from the biocontrol bacterium *Pseudomonas fluorescens*. *Phytochemistry*, 62:1105–1114.
78. Pesci, E.C., Milbank, J.B., Pearson, J.P., McKnight, S., Kende, A.S., Greenberg, E.P., and Iglewski, B.H., 1999, Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA*, 96:11229–11234.
79. Pesci, E.C., Pearson, J.P., Seed, P.C., and Iglewski, B.H., 1997, Regulation of las and rhl quorum sensing in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 179:3127–3132.
80. Pessi, G., Williams, F., Hindle, Z., Heurlier, K., Holden, M.T., Camara, M., Haas, D., and Williams, P., 2001, The global posttranscriptional regulator RsmA modulates production of virulence determinants and N-acylhomoserine lactones in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 183:6676–6683.
81. Picard, C. and Bosco, M., 2003, Genetic diversity of *phlD* gene from 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp. strains from the maize rhizosphere. *FEMS Microbiol. Lett.*, 219:167–172.
82. Picard, C., Di Cello, F., Ventura, M., Fani, R., and Guckert, A., 2000, Frequency and biodiversity of 2,4-diacetylphloroglucinol-producing bacteria isolated from the maize rhizosphere at different stages of plant growth. *Appl. Environ. Microbiol.*, 66:948–955.
83. Pierson, L.S., III, Wood, D.W., and Pierson, E.A., 1998, Homoserine lactone-mediated gene regulation in plant-associated bacteria. *Annu. Rev. Phytopathol.*, 36:207–225.
84. Pierson, L.S., III, Gaffney, T., Lam, S., and Gong, F., 1995, Molecular analysis of genes encoding phenazine biosynthesis in the biological control bacterium *Pseudomonas aureofaciens* 30-84. *FEMS Microbiol. Lett.*, 134:299–307.

85. Pierson, L.S., III, Keppenne, V.D., and Wood, D.W., 1994, Phenazine antibiotic biosynthesis in *Pseudomonas aureofaciens* 30–84 is regulated by PhzR in response to cell density. *J. Bacteriol.*, 176:3966–3974.
86. Pierson, L.S., III and Thomashow, L.S., 1992, Cloning and heterologous expression of the phenazine biosynthetic locus from *Pseudomonas aureofaciens* 30–84. *Mol. Plant Microbe Interact.*, 5:330–339.
87. Poole, K. and McKay, G.A., 2003, Iron acquisition and its control in *Pseudomonas aeruginosa*: Many roads lead to Rome. *Front Biosci.*, 8:D661–D686.
88. Raaijmakers, J.M., Vlami, M., and de Souza, J.T., 2002, Antibiotic production by bacterial biocontrol agents. *Antonie Van Leeuwenhoek*, 81:537–547.
89. Raaijmakers, J.M. and Weller, D.M., 2001, Exploiting genotypic diversity of 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp.: Characterization of superior root-colonizing *P. fluorescens* strain Q8r1-96. *Appl. Environ. Microbiol.*, 67:2545–2554.
90. Raaijmakers, J.M., Weller, D.M., and Thomashow, L.S., 1997, Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments. *Appl. Environ. Microbiol.*, 63:881–887.
91. Ramette, A., Moenne-Loccoz, Y., and Defago, G., 2001, Polymorphism of the polyketide synthase gene *phlD* in biocontrol fluorescent pseudomonads producing 2,4-diacetylphloroglucinol and comparison of PhlD with plant polyketide synthases. *Mol. Plant Microbe Interact.*, 14:639–652.
92. Ramette, A., Moenne-Loccoz, Y., and Defago, G., 2003, Prevalence of fluorescent pseudomonads producing antifungal phloroglucinols and/or hydrogen cyanide in soils naturally suppressive or conducive to tobacco black root rot. *FEMS Microbiol. Ecol.*, 44:35–43.
93. Reimmann, C., Beyeler, M., Latifi, A., Winteler, H., Foglino, M., Lazdunski, A., and Haas, D., 1997, The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer N-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. *Mol. Microbiol.*, 24:309–319.
94. Rumbaugh, K.P., Griswold, J.A., and Hamood, A.N., 2000, The role of quorum sensing in the in vivo virulence of *Pseudomonas aeruginosa*. *Microbes Infect.*, 2:1721–1731.
95. Salmond, G.P., Bycroft, B.W., Stewart, G.S., and Williams, P., 1995, The bacterial “enigma”: Cracking the code of cell–cell communication. *Mol. Microbiol.*, 16:615–624.
96. Schnider, U., Keel, C., Blumer, C., Troxler, J., Defago, G., and Haas, D., 1995, Amplification of the housekeeping sigma factor in *Pseudomonas fluorescens* CHA0 enhances antibiotic production and improves biocontrol abilities. *J. Bacteriol.*, 177:5387–5392.
97. Schnider-Keel, U., Seematter, A., Maurhofer, M., Blumer, C., Duffy, B., Gigot-Bonnefoy, C., Reimmann, C., Notz, R., Defago, G., Haas, D., and Keel, C., 2000, Autoinduction of 2,4-diacetylphloroglucinol biosynthesis in the biocontrol agent *Pseudomonas fluorescens* CHA0 and repression by the bacterial metabolites salicylate and pyoluteorin. *J. Bacteriol.*, 182:1215–1225.
98. Seedorf, U., Brysch, P., Engel, T., Schrage, K., and Assmann, G., 1994, Sterol carrier protein X is peroxisomal 3-oxoacyl coenzyme A thiolase with intrinsic sterol carrier and lipid transfer activity. *J. Biol. Chem.*, 269:21277–21283.
99. Shanahan, P., O’Sullivan, D., Simpson, P., Glennon, J.D., and O’ Gara, F.F., 1992, Isolation and characterisation of an antibiotic like compound from a fluorescent pseudomonad and investigation of physiological parameters influencing its production. *Appl. Environ. Microbiol.*, 58:353–358.
100. Sitnikov, D.M., Schineller, J.B., and Baldwin, T.O., 1995, Transcriptional regulation of bioluminescence genes from *Vibrio fischeri*. *Mol. Microbiol.*, 17:801–812.
101. Smith, R.S. and Iglewski, B.H., 2003, *P. aeruginosa* quorum-sensing systems and virulence. *Curr. Opin. Microbiol.*, 6:56–60.

102. Sorensen, D., Nielsen, T.H., Christophersen, C., Sorensen, J., and Gajhede, M., 2001, Cyclic lipoundecapeptide amphisin from *Pseudomonas* sp. strain DSS73. *Acta Crystallogr. C*, 57:1123–1124.
103. Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrenner, P., Hickey, M.J., Brinkman, F.S., Hufnagle, W.O., Kowalik, D.J., Lagrou, M., Garber, R.L., Goltry, L., Tolentino, E., Westbrook-Wadman, S., Yuan, Y., Brody, L.L., Coulter, S.N., Folger, K.R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G.K., Wu, Z., Paulsen, I.T., Reizer, J., Saier, M.H., Hancock, R.E., Lory, S., and Olson, M.V., 2000, Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature*, 406:959–964.
104. Suzuki, K., Wang, X., Weilbacher, T., Pernestig, A.K., Melefors, O., Georgellis, D., Babitzke, P., and Romeo, T., 2002, Regulatory circuitry of the CsrA/CsrB and BarA/UvrY systems of *Escherichia coli*. *J. Bacteriol.*, 184:5130–5140.
105. Timms-Wilson, T.M., Ellis, R.J., Renwick, A., Rhodes, D.J., Mavrodi, D.V., Weller, D.M., Thomashow, L.S., and Bailey, M.J., 2000, Chromosomal insertion of phenazine-1-carboxylic acid biosynthetic pathway enhances efficacy of damping-off disease control by *Pseudomonas fluorescens*. *Mol. Plant Microbe Interact.*, 13:1293–1300.
106. Ton, J., De Vos, M., Robben, C., Buchala, A., Mettraux, J.P., Van Loon, L.C., and Pieterse, C.M., 2002, Characterization of Arabidopsis enhanced disease susceptibility mutants that are affected in systemically induced resistance. *Plant J.*, 29:11–21.
107. Tsay, J.-T., Ho, W., Larson, T.J., Jackowski, Y., and Rock, C.O., 1992, Isolation and characterisation of the b-ketoacyl carrier protein synthase III gene (*fabH*) from *Escherichia coli* K-12. *J. Biol. Chem.*, 267:6807–6814.
108. Turner, J.M. and Messenger, A.J., 1986, Occurrence, biochemistry and physiology of phenazine pigment production. *Adv. Microb. Physiol.*, 27:211–275.
109. Van Loon, L.C., Bakker, P.A.H.M., and Pieterse, C.M.J., 1998, Systemic resistance induced by rhizosphere bacteria. *Annu. Rev. Phytopathol.*, 35:453–483.
110. van Pee, K.H. and Ligon, J.M., 2000, Biosynthesis of pyrrolnitrin and other phenylpyrrole derivatives by bacteria. *Nat. Prod. Rep.*, 17:157–164.
111. van Wees, S.C., Luijendijk, M., Smoorenburg, I., van Loon, L.C., and Pieterse, C.M., 1999, Rhizobacteria-mediated induced systemic resistance (ISR) in Arabidopsis is not associated with a direct effect on expression of known defense-related genes but stimulates the expression of the jasmonate-inducible gene *Atvsp* upon challenge. *Plant Mol. Biol.*, 41:537–549.
112. Vance, C.P., 2001, Symbiotic nitrogen fixation and phosphorus acquisition. Plant nutrition in a world of declining renewable resources. *Plant Physiol.*, 127:390–397.
113. Vincent, M.N., Harrison, L.A., Brackin, J.M., Kovacevich, P.A., Mukerji, P., Weller, D.M., and Pierson, E.A., 1991, Genetic analysis of the antifungal activity of a soilborne *Pseudomonas aureofaciens* strain. *Appl. Environ. Microbiol.*, 57:2928–2934.
114. Walsh, U.F., Morrissey, J.P., and O’Gara, F., 2001, *Pseudomonas* for biocontrol of phytopathogens: From functional genomics to commercial exploitation. *Curr. Opin. Biotechnol.*, 12:289–295.
115. Wang, C., Ramette, A., Punjasamarnwong, P., Zala, M., Natsch, A., Moenne-Loccoz, Y., and Defago, G., 2001, Cosmopolitan distribution of *phlD*-containing dicotyledonous crop-associated biocontrol pseudomonads of worldwide origin. *FEMS Microbiol. Ecol.*, 37:105–116.
116. Weller, D.M., Raaijmakers, J.M., Gardener, B.B., and Thomashow, L.S., 2002, Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annu. Rev. Phytopathol.*, 40:309–348.
117. Whistler, C.A., Corbell, N.A., Sarniguet, A., Ream, W., and Loper, J.E., 1998, The two-component regulators GacS and GacA influence accumulation of the stationary-phase sigma factor sigmaS and the stress response in *Pseudomonas fluorescens* Pf-5. *J. Bacteriol.*, 180:6635–6641.

118. Whitehead, I.N. and Dixon, R.A., 1983, Chalcone synthase from cell suspension cultures of *Phaseolus vulgaris* L. *Biochem. Biophys. Acta.*, 747:298–303.
119. Wisniewski-Dye, F. and Downie, J.A., 2002, Quorum-sensing in *Rhizobium*. *Antonie Van Leeuwenhoek*, 81:397–407.
120. Woo, S., Fogliano, V., Scala, F., and Lorito, M., 2002, Synergism between fungal enzymes and bacterial antibiotics may enhance biocontrol. *Antonie Van Leeuwenhoek*, 81:353–356.
121. Wood, D.W., Gong, F., Daykin, M.M., Williams, P., and Pierson, L.S., III, 1997, N-acyl-homoserine lactone-mediated regulation of phenazine gene expression by *Pseudomonas aureofaciens* 30–84 in the wheat rhizosphere. *J. Bacteriol.*, 179:7663–70.
122. Wood, D.W. and Pierson, L.S., III, 1996, The *phzI* gene of *Pseudomonas aureofaciens* 30–84 is responsible for the production of a diffusible signal required for phenazine antibiotic production. *Gene*, 168:49–53.

CYANOGENESIS

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1. INTRODUCTION

The production of hydrogen cyanide (HCN) by *Pseudomonas aeruginosa* and other fluorescent pseudomonads was discovered by Clawson and Young in 1913 when these authors identified, for the first time, cyanogenic bacteria isolated from different habitats¹⁸. Although the physiological conditions favoring HCN formation were not investigated in detail, the presence of oxygen was recognized as being important¹⁸. A further critical observation was made by Friedheim in 1934: he noted that the aerobic bacterium *P. aeruginosa* “lives almost as an anaerobic organism even in the presence of air.”²⁷ As it turned out later, HCN is produced optimally by *P. aeruginosa* under microaerobic conditions^{10, 13, 14}, to which this bacterium is well adapted⁶¹. In 1948, Lorck found glycine to be the metabolic precursor of HCN in *P. aeruginosa*⁴⁵. Biochemical details of the glycine-to-HCN conversion, worked out mostly by the groups of Wissing and Castric in the 1970s^{12, 82}, were obtained in whole cells and cell

extracts of two different pseudomonads. Due to technical difficulties, the proteins catalyzing HCN biosynthesis in pseudomonads have never been purified to homogeneity. Thus, the enzymatic mechanism and the structural model of HCN synthase, which are deduced, in part, from a genetic analysis of the *hcn* structural genes in *Pseudomonas fluorescens* CHA0 and *P. aeruginosa* PAO (discussed below), should be considered as working hypotheses. HCN formation from glycine appears to be restricted to strains of *Pseudomonas* spp. and *Chromobacterium violaceum*^{12, 38}. Other pathways producing cyanide occur in *Escherichia coli* and in some cyanobacteria, fungi, algae and plants^{35, 38, 59, 71}.

At physiological pH and in the absence of complexing ions, HCN (pK_a 9.3) is largely undissociated and volatile. HCN and cyanide (CN⁻), which we do not discriminate in this chapter, are highly toxic to most organisms, because of potent inhibition of many enzymes containing heme-copper, molybdenum or zinc ligands⁶⁵. A simple and specific bioassay for the toxicity of cyanide produced by *P. aeruginosa* is based on the nematode *Caenorhabditis elegans* which is paralyzed and killed within less than 4 h when it crawls over a lawn of HCN-positive *P. aeruginosa*²⁸. Like many antibiotics of bacterial or fungal origin, HCN is produced by *Pseudomonas* spp. as a typical secondary metabolite^{10, 12}. This implies that strains producing HCN are resistant to HCN at the levels produced^{12, 68}.

2. HCN SYNTHASE

In *P. fluorescens* CHA0 and in *P. aeruginosa* PAO, three HCN biosynthetic genes (*hcnABC*) are clustered and probably constitute an operon^{44, 51}. Mutational inactivation of the *hcnABC* genes causes loss of cyanogenesis in both bacterial species^{44, 51}. It is therefore unlikely that alternative HCN biosynthetic pathways exist in these bacteria, such as the histidine oxidation pathway present in some cyanobacteria⁷¹ or the HypEF-catalyzed reactions of *E. coli*, which uses carbamoylphosphate as the precursor to deliver cyanide to the active center NiFe-hydrogenase⁵⁹. Artificial expression of the *P. fluorescens* *hcnABC* cluster in *E. coli* leads to massive and lethal HCN production in this host, suggesting that these genes are sufficient to encode an enzymatic complex catalyzing the formation of HCN⁴⁴. This putative complex is designated HCN synthase¹⁶.

The biochemical properties of HCN synthase can be summarized as follows. Glycine is stoichiometrically oxidized to HCN and CO₂ in a reaction which conserves the substrate's C–N bond in HCN and which derives CO₂ from the carboxyl group of glycine (Figure 1). FAD stimulates the

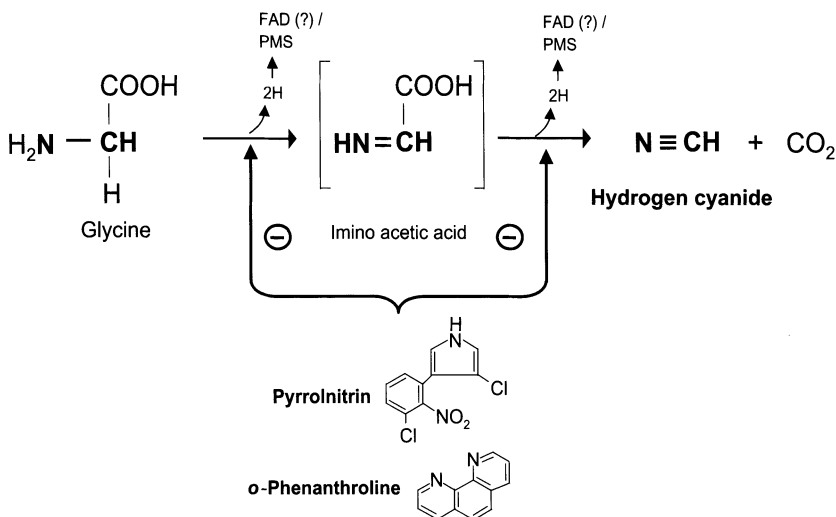


Figure 1. HCN synthase reaction according to the glycine dehydrogenase model⁷⁹. In each of the two reactions shown, 2 H are transferred to the putative FAD coenzyme of HCN synthase, then 2e⁻ are transferred either to the respiratory chain in vivo or to an artificial e⁻ acceptor such as PMS in vitro. Pyrrolnitrin and o-phenanthroline are potent inhibitors of the reaction.

reaction in vitro, whereas pyrrolnitrin and acriflavin, inhibitors of flavin enzymes, and o-phenanthroline, a strong iron chelator, inhibit markedly the synthesis of HCN (Figure 1). In vitro, an artificial electron acceptor such as phenazine methosulfate (PMS) is needed to drive the reaction. In vivo, the electron acceptor is oxygen, involving unidentified components of the respiratory chain^{11, 12, 14, 79, 80, 82}. By analogy with the glycine oxidase reaction, catalyzed by the FAD-dependent ThiO (GoxB) enzyme of thiamine biosynthesis in *Bacillus subtilis*, it is postulated that hydride transfer from glycine to the flavin moiety of the coenzyme produces iminoacetic acid as an intermediate of the HCN synthase reaction^{48, 64}. ThiO and HcnC of *P. fluorescens* and *P. aeruginosa* share 28% and 26% of amino acid sequence identity, respectively. Unfortunately, iminoacetic acid is too unstable to be handled. In conclusion, the available biochemical data suggest that HCN synthase is a flavoenzyme, contains one or several iron cofactors, and acts by transferring 4 H to the respiratory chain (Figures 1 and 2). As a systematic name for HCN synthase, glycine dehydrogenase (cyanide-forming; EC 1.4.99.-) has been proposed⁷.

The deduced amino acid sequences of the HcnA, HcnB and HcnC proteins of *P. fluorescens* and *P. aeruginosa* have 69%, 70% and 75% identity

with each other, respectively⁵¹. Therefore, sequence motifs previously detected in the HcnABC proteins of *P. fluorescens*^{7, 44} can also be found in the HcnABC complex of *P. aeruginosa*. In HcnA, there is a [2Fe–2S] binding motif with four characteristic cysteine residues⁷. Using the Prosite and Pfam databases (ref. [5]; <http://www.expasy.org/prosite>; <http://pfam.wustl.edu>; <http://hits.isb-sib.ch>), we also recognize a [2Fe–2S] binding domain (which we had not identified previously) in the C-terminal part of HcnB. Both putative [2Fe–2S] centers in HCN synthase are of the ferredoxin-type and they could account for the fact that the enzyme is destabilized by exposure to oxygen^{14, 81} and inhibited by *o*-phenanthroline⁸⁰; earlier studies have demonstrated that the iron-sulfur cluster of ferredoxin slowly reacts with the Fe²⁺ chelating agent *o*-phenanthroline⁴⁶. HcnB and HcnC each contain a typical FAD/NAD-binding motif with a predicted βαβ-fold, in their N-terminal parts^{44, 76}. A BLAST search for conserved domains in the NCBI database reveals similarities of HcnB with the pyridine nucleotide-disulfide oxidoreductase family (pfam 00070) and of HcnC with a family of deaminating glycine/D-amino acid oxidases (dehydrogenases; pfam 01266), whereas HcnA resembles the NuoG subunit of NADH dehydrogenase (COG1034; <http://www.ncbi.nlm.nih.gov>)^{24, 66}. These features lend support to a dehydrogenase mechanism of HCN synthase, similar to that of D-amino acid dehydrogenase^{49, 64}. Furthermore, nopaline oxidase and octopine oxidase of *Agrobacterium tumefaciens* both appear to be encoded by three-gene clusters similar to *hcnABC*^{7, 83}. However, an important difference should be pointed out. The imino acids produced by D-amino acid oxidases and opine oxidases are hydrolyzed immediately, resulting in cleavage of the C–N bond and producing a 2-keto acid^{7, 32}. By contrast, in ThiO-initiated thiamine biosynthesis as well as in the HCN synthase reaction, the C–N bond is conserved^{64, 81}. The mechanism by which the C–C bond of glycine is cleaved by HCN synthase remains a matter of speculation^{7, 44}.

HCN synthase from a *Pseudomonas* sp., when solubilized with the non-ionic detergent Triton X-100, has higher activity, compared to enzyme preparations obtained by sonication⁸². Thus, HCN synthase may be anchored in the cytoplasmic membrane. Four different methods^{36, 37, 41, 56} all predict two transmembrane helices in the N-terminal part of HcnC, although their previously proposed locations⁴⁴ are not certain. For HcnB, two methods^{37, 56} also suggest two potential transmembrane segments⁴⁴, but more recent analyses using two new algorithms^{36, 41} fail to reveal any transmembrane topology and, therefore, HcnB is more likely to be a peripheral membrane protein. The small HcnA subunit apparently does not have any transmembrane helix. From these considerations, we propose a structural model for HCN synthase (Figure 2), which arbitrarily assumes a 1:1:1 subunit

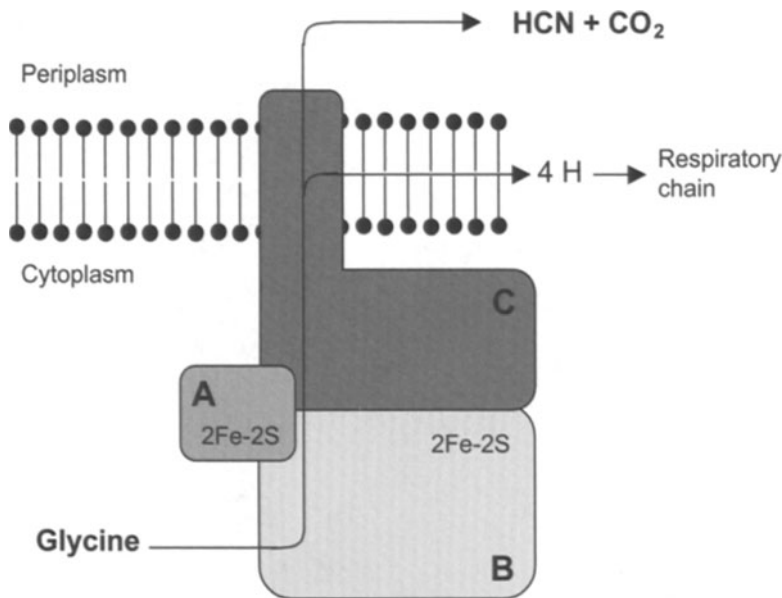


Figure 2. Hypothetical model of HCN synthase in fluorescent pseudomonads. The enzyme consists of subunit A (11.5 kDa), subunit B (50.6 kDa) and subunit C (45.3 kDa). The properties of HCN synthase are described in the text.

composition. The model postulates that the products (HCN, CO_2) are released into the bacterium's periplasm.

3. REGULATION OF HCN SYNTHASE EXPRESSION

Due to oxygen-sensitivity and instability of HCN synthase, most expression studies have been based on measurements of HCN accumulated in bacterial cultures rather than on determinations of specific HCN synthase activities^{12, 82}. From HCN accumulation data, one can calculate the cyanogenic capacity of cells during growth¹⁵; however, factors regulating HCN synthase formation cannot be discriminated from those influencing enzyme activity by this approach. Isolation of the *hcnABC* structural genes from *P. fluorescens* and *P. aeruginosa* has allowed the construction of transcriptional and translational *hcnA-lacZ* fusions^{44, 51}. These are suited to assess transcriptional and post-transcriptional regulation of HCN synthase expression^{8, 9, 33, 51-53}. By contrast, the mechanisms that terminate the expression of the *hcn* genes are unknown, but clearly very important for the HCN producer and its environment¹⁶.

3.1. Oxygen

In *P. aeruginosa*, dissolved oxygen concentrations of $<20\ \mu\text{M}$ in the culture medium induce cyanogenesis^{13, 39}. This effect is mediated by the transcriptional regulator ANR⁸⁴, which enhances *hcnABC* transcription about 6-fold by binding to a palindromic sequence known as the ANR box, located upstream of the *hcn* promoter⁵¹. In *P. fluorescens* CHA0, the inducing effect of ANR is >50 -fold⁸. This difference can be explained by the fact that the ANR box in the *hcn* promoter of *P. fluorescens* (TTGGCN₄ATCAA) is more similar to the ANR consensus (TTGATN₄ATCAA) than is the ANR box in the *hcn* promoter of *P. aeruginosa* (CTGTCN₄ATCAA)^{44, 51, 78}. ANR is closely related to the O₂ sensor-regulator FNR of *E. coli*, in terms of structure and mechanism^{2, 63, 84}. In *P. fluorescens*, half-maximal activity of ANR occurs at about $5\ \mu\text{M O}_2$ ³⁴. The same value has been reported for FNR in *E. coli*². Under fully aerobic conditions, FNR and ANR are inactivated, following oxidation of their [4Fe–4S] cofactor and dissociation of the dimeric transcriptional activator into a monomeric form². Strong aeration (i.e., $180\ \mu\text{M O}_2$) also leads to complete and irreversible inactivation of HCN synthase in *P. aeruginosa*, with a half-life of 10 min. The substrate glycine protects the enzyme from this oxidative inactivation^{13, 16}. Under strictly anaerobic, denitrifying conditions, *P. aeruginosa* PAO grows well but does not produce HCN¹², whereas *P. fluorescens* CHA0 ceases both growth and cyanogenesis³⁴. In summary, cyanogenesis is optimal in the vicinity of $2\ \mu\text{M O}_2$ (corresponding to about 1% air saturation) in *Pseudomonas* spp.^{12, 13}, because microaerobic conditions induce transcription of the *hcnABC* cluster, allow electron transfer from HCN synthase to O₂ via components of the respiratory chain, and minimize inactivation of the enzyme.

3.2. Iron

In both *P. fluorescens* and *P. aeruginosa*, moderate concentrations of Fe³⁺ (e.g., $20\ \mu\text{M}$) in the growth medium stimulate cyanogenesis markedly^{3, 8, 10, 72}. The ANR box in the *hcn* promoter and the ANR protein are needed to bring about this inducing effect in strain CHA0⁸. Presumably, conditions of iron sufficiency favor the assembly of the [4Fe–4S] cofactor of ANR⁸. On the other hand, iron limitation not only impedes ANR function but also interferes with the activity of HCN synthase⁷⁹.

3.3. Quorum Sensing

In *P. aeruginosa*, optimal HCN production in batch cultures occurs during the transition from exponential growth to stationary phase, at high cell

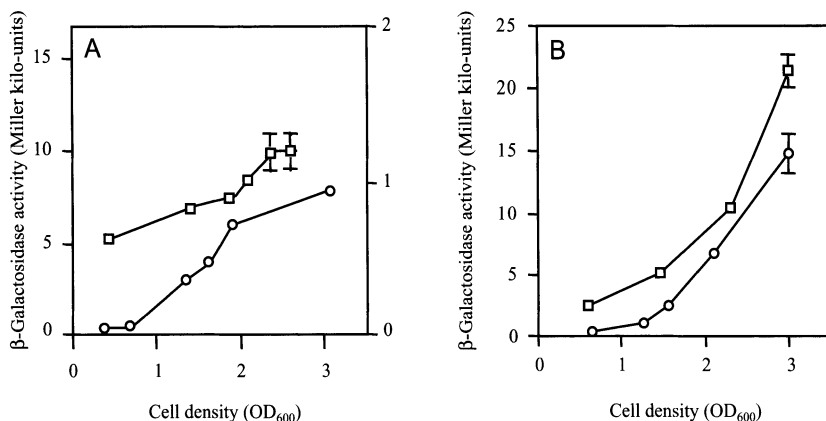


Figure 3. Cell density-dependent *hcnA* expression in *P. aeruginosa* PAO and *P. fluorescens* CHA0 growing in nutrient yeast broth under mild oxygen limitation. β -Galactosidase activities of plasmid-borne translational *hcnA'*-*'lacZ* fusions driven from the *hcn* promoter (circles) or from the *tac* promoter (squares) were determined in (A) *P. aeruginosa* with pME3826 (*hcnA'*-*'lacZ*) and pME3843 (*ptac-hcnA'*-*'lacZ*), respectively, and (B) *P. fluorescens* with pME3219 (*hcnA'*-*'lacZ*) and pME6530 (*ptac-hcnA'*-*'lacZ*), respectively^{9, 51, 52}. In A, the activity of the *hcnA'*-*'lacZ* fusion of pME3826 is reported on the right y-axis. Cell densities are expressed as optical densities (OD) at 600 nm.

densities¹⁵. This expression pattern is typical of quorum sensing regulation (see Chapter 1, Volume 2). Indeed, both quorum sensing regulators LasR and RhIR, together with their cognate signals *N*-(3-oxododecanoyl)-homoserine lactone (OdDHL) and *N*-butanoyl-homoserine lactone (BHL), respectively, are required for cyanogenesis^{58, 75, 77}. Cell density-dependent expression of an *hcnA'*-*'lacZ* translational fusion in *P. aeruginosa* PAO is illustrated in Figure 3A. When the native *hcn* promoter is replaced by a constitutive promoter (*ptac*), expression of the *hcnA'*-*'lacZ* construct is much less dependent on the cell density (Figure 3A), suggesting that most (but not all) of quorum sensing control of cyanogenesis involves the *hcn* promoter. Genetic analysis of this promoter⁵¹ has revealed two transcription start sites (T1, T2) separated by 29 bp (Figure 4). Transcription from T2 is activated by three regulatory proteins: LasR and RhIR (they interact with the LUX box at -71.5 bp, probably as a heterodimer) as well as ANR (it interacts with the ANR box at -42.5 bp) (Figure 4). However, the orientation of the putative LasR-RhIR heterodimer has not been determined. An additional, less conserved LUX box (not shown in Figure 4) is located 190 bp upstream of T2 and makes a minor positive contribution as an enhancer to *hcn* expression. Thus, this promoter architecture permits synergistic transcriptional activation by LasR, RhIR and ANR in the presence of OdDHL and BHL, and in a micro-aerobic environment⁵¹. When ANR is inactive, transcription can occur from the upstream T1 site, controlled only by LasR and RhIR⁵¹.

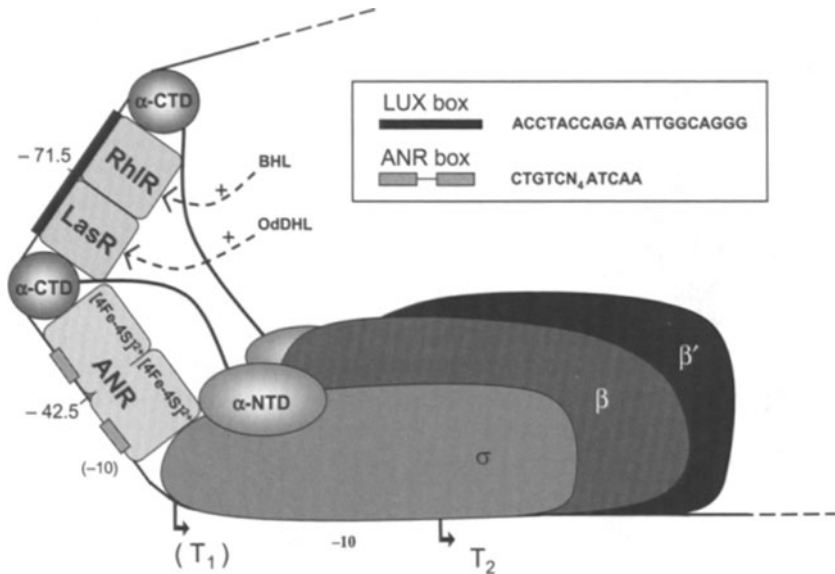


Figure 4. Model for the recognition of the *hcn* promoter by the transcriptional regulators ANR, LasR and RhIR and their interaction with RNA polymerase in *P. aeruginosa*. α -CTD (C-terminal domain of the α subunit) and α -NTD (N-terminal domain of the α subunit) of RNA polymerase are shown as separate domains joined by a linker. Contacts of α -CTD with upstream promoter sequences have not been determined experimentally, but are inferred from a study on similarly activated *E. coli* promoters⁶. The positions relative to the transcriptional start site T2 are indicated below the recognition sequences. During oxygen limitation, ANR blocks transcription from site T1 by binding to the corresponding -10 sequence (in brackets). During good aeration, ANR is inactive and the promoter is under LasR/RhIR control only, with transcription initiation at T1. Adapted from ref. [51].

Cell density-dependent expression of an *hcnA'*-*lacZ* fusion is also observed in *P. fluorescens* CHA0 (Figure 3B). Intriguingly, most of this regulation persists after replacement of the *hcn* promoter by the *tac* promoter (Figure 3B), suggesting that in this organism quorum sensing control operates in a different way and at a post-transcriptional level. Strain CHA0 has no LUX box in its *hcn* promoter and apparently does not produce *N*-acyl-homoserine lactone signals^{31, 33, 85}. When the 5' *hcnA* leader region is removed from the *ptac-hcnA'*-*lacZ* construct, cell density-dependent regulation is lost^{9, 85}. The *hcnA* leader contains a target of the global GacS/GacA signal transduction pathway^{9, 43}. This target consists of *c.* 12 nucleotides located at and around the *hcnA* ribosome binding site (RBS) and is essential for regulation by the GacS/GacA system⁹ (K. Starke and D. Haas, unpublished results). At least two small RNA-binding proteins, RsmA^{9, 33} and RsmE³¹, are assumed to interact

with this target, causing post-transcriptional repression of cyanogenesis. The GacS/GacA two-component system upregulates the expression of two small, non-coding RNAs, RsmY⁶⁹ and RsmZ³³, at the end of exponential growth. RsmY and RsmZ antagonize the repressive action of RsmA and RsmE on *hcnA* and other target genes, by sequestering the RNA-binding proteins in RNA-protein complexes. In support of this model, overexpression of the *rsmZ* gene in *P. fluorescens* CHA0 and of the closely related *prfB* gene in *P. fluorescens* F113 has a stimulating effect on cyanogenesis^{1, 33} and loss of *gacS/gacA* function results in the loss of HCN production in *P. fluorescens* CHA0, *P. fluorescens* F113 and *P. aureofaciens* 30-84^{17, 43, 62}. Details of this signal transduction pathway, which appears to be widespread in Gram-negative bacteria, are described by Heeb *et al.* in Volume 2, Chapter 8. At the end of exponential growth, *P. fluorescens* CHA0 and many other pseudomonads produce low-molecular-weight signal molecules which are not related to *N*-acyl-homoserine lactones and remain to be identified chemically³³ (C. Dubuis and D. Haas, unpublished results). When added to growing cultures of *P. fluorescens*, these signal molecules stimulate several-fold the expression of the *rsmY* and *rsmZ* genes and hence that of target genes such as *hcnA*^{33, 69}. In summary, cell density-related stimulation of *hcnA*'-'*lacZ* expression in *P. fluorescens* CHA0 (Figure 3B) is essentially controlled by the GacS/GacA regulatory cascade.

In *P. aeruginosa*, the GacS/GacA system exerts a dual control on the expression of the HCN synthase genes. First, the signal transduction pathway described above for *P. fluorescens* provides similar post-transcriptional control of *hcnA* expression also in *P. aeruginosa*^{52, 53}, although the induction factor is smaller in *P. aeruginosa* than in *P. fluorescens*, as is evident from the expression pattern of the *lacZ* fusions specified by pME3843 and pME6530, respectively (Figure 3A and B). Second, the GacS/GacA system upregulates components of the *N*-acyl-homoserine lactone-driven quorum sensing machinery in *P. aeruginosa* and thereby indirectly has a positive effect on *hcnABC* expression at the level of transcription^{51, 58}.

3.4. Other Regulatory Elements

Glycine added in millimolar amounts to the growth medium stimulates HCN production in several isolates of fluorescent pseudomonads^{11, 12, 15, 45, 72, 82}; in strain CHA0, glycine does not enhance *hcnA*'-'*lacZ* expression⁸. The most likely explanation for the positive effect of glycine is that this compound stabilizes HCN synthase and acts as the substrate for the enzyme¹⁶. Methionine and inorganic phosphate, added in millimolar concentrations as growth supplements, also have positive effects on HCN production in *P. aeruginosa*, via unknown mechanisms^{11, 12}. The *Pseudomonas* quinolone signal (PQS), 2-heptyl-3-hydroxy-4-quinolone, is part of the quorum sensing

regulatory network of *P. aeruginosa*; LasR is required for PQS synthesis⁵⁰. PQS-negative mutants (e.g., *pqsH* or *pqsR*) are downregulated for *hcnB-lacZ* transcription and thus produce significantly reduced amounts of HCN, compared to the *P. aeruginosa* wild type^{28, 29}. Similarly, mutation of a zinc uptake regulator gene (*zur*) leads to low PQS levels, poor *hcnB-lacZ* expression and reduced virulence in neutropenic mice²⁹. In *P. aeruginosa*, a homolog of the BvgS sensor kinase, which is required for *Bordetella* virulence gene regulation, also participates in the regulation of cyanogenesis. A *bvgS*-like (PA3946) mutant is strongly affected in *hcnB-lacZ* expression and HCN production^{28, 29}. Furthermore, a *mucA* mutant of *P. aeruginosa*, in which the alternative sigma factor σ^E (= AlgU = AlgT) is activated, is induced about 5-fold for *hcnABC* transcription²⁶. How methionine, phosphate, PQS, the BvgS-like regulator and σ^E regulate cyanogenesis is not known at present.

4. CYANIDE RESISTANCE OF CYANOGENIC PSEUDOMONADS

Why do HCN producers not kill themselves? *P. aeruginosa* has four terminal oxidases of the heme-copper superfamily, as predicted from the total genomic sequence: a *bo₃*-type quinol oxidase, two *cbb₃*-type cytochrome oxidases and an *aa₃*-type cytochrome oxidase^{19, 20}. All these components of the aerobic respiratory chain are expected to be sensitive to cyanide at levels (*c.* 0.1 mM) typically found in microaerobic, dense *P. aeruginosa* cultures. However, *P. aeruginosa* has a fifth, cyanide-insensitive cytochrome oxidase encoded by the *cioAB* genes^{19, 47, 68}, which does not contain a heme-copper center and is a *bd*-type quinol oxidase^{21, 67}.

The *cioAB* genes are inducible by low oxygen concentrations and by cyanide at 0.1 mM^{19–21}. *P. aeruginosa* mutants defective in the RoxSR two-component system show reduced *cio-lacZ* expression and are no longer inducible by cyanide. Thus, these mutants are more sensitive to cyanide than is the wild type. The response regulator RoxR binds to the *cio* promoter and is postulated to function as a transcriptional activator of the *cioAB* genes under the control of the RoxS sensor, which responds directly or indirectly to cyanide¹⁹. ANR turns out to be a repressor of the *cio* promoter^{20, 21}. More work will be needed to establish the mechanisms by which low oxygen concentrations and the electron flow through the cyanide-sensitive branches of the respiratory chain coordinate the expression of the *cioAB* genes and the production of HCN.

Although not supported by mutant studies, detoxifying mechanism may contribute to the cyanide tolerance of *Pseudomonas* spp. Cyanide detoxifying mechanisms are widespread in nature^{38, 57}. One such mechanism provided by

rhodanese ($\text{S}_2\text{O}_3^{2-} + \text{CN}^- \rightarrow \text{SCN}^- + \text{SO}_3^{2-}$) may also exist in *P. aeruginosa*, which produces this enzyme constitutively in the cytoplasm⁶⁰. Cyanide penetrating into the cytoplasm might be scavenged and neutralized by rhodanese. Among several nonenzymatic mechanisms for cyanide detoxification, the base-catalyzed formation of cyanohydrins from aldehydes (including reducing sugars) and reactive ketones ($\text{R-CO-R}' + \text{HCN} \rightleftharpoons \text{R-C(OH)(CN)-R}'$) can have biological significance. For instance, extracellular accumulation of pyruvate and 2-ketoglutarate in *P. fluorescens* NCIMB11764 cultures grown under nitrogen-limiting conditions, detoxifies cyanide by cyanohydrin formation. Subsequently this strain degrades the cyanohydrin intracellularly, resulting in the utilization of cyanide as a nitrogen source⁴². *P. aeruginosa* also excretes 2-ketoglutarate under nitrogen limitation⁷⁴ and thus may have the capacity to buffer against high extracellular cyanide concentrations. A related effect can be seen in *proC* mutants of *P. aeruginosa* which excrete glutamic-5-semialdehyde (Δ^1 -pyrroline-5-carboxylate) and thereby detoxify the HCN produced²⁸.

5. ECOLOGY OF HCN PRODUCTION

In the bacterial world, the production and release of substantial amounts of HCN (typically 0.1–0.2 mM, maximally about 1 mM) into the growth medium is confined to fluorescent pseudomonads (*P. aeruginosa*, *P. fluorescens*, *Pseudomonas chlororaphis*, *Pseudomonas aureofaciens*, and related species) and to *C. violaceum*^{12, 38}. Sequence analysis of the *hcnBC* genes from cyanogenic, plant-associated fluorescent pseudomonads reveals four distinct Hcn groups. These groups do not coincide with taxonomic groups based of 16S rRNA phylogeny, suggesting that perhaps the *hcnABC* genes have been horizontally transferred between *Pseudomonas* spp.⁵⁴.

HCN has several attributes of a typical, bacterial secondary metabolite: no apparent function in primary metabolism, production during restricted growth, tolerance of the producer organism to HCN, and possible roles in the ecological niche of the producer^{7, 10}. Does cyanogenesis confer a selective advantage on the producer? This question is still open. By analogy with cyanogenic plants, which use cyanide as a chemical defense strategy against herbivores³⁵, it can be argued that bacteria producing HCN may be protected from predation. In the *P. aeruginosa*–*C. elegans* biotest, bacterial HCN first paralyses, then kills the nematodes²⁸. In this interaction, HCN qualifies as a virulence factor. Cyanide has been detected in wounds infected with *P. aeruginosa*³⁰, thus a role of cyanide as a virulence factor in infected animal and human tissues is a strong possibility, especially under conditions of low oxygen availability.

A number of plant-deleterious rhizobacteria belonging to *Pseudomonas* spp. are HCN producers and it has been proposed, though without solid experimental evidence, that the negative effects of such bacteria on plant growth could be mediated by HCN^{4, 22, 40, 70}. By contrast, the fact that HCN formed by plant-associated bacteria can be beneficial to plants is well documented by the use of genetically modified biocontrol bacteria in microcosm experiments. *P. fluorescens* CHA0 mutants impaired in HCN production have a reduced ability to protect tobacco roots from the fungal pathogen *Thielaviopsis basicola*, compared to the biocontrol ability of the wild type^{44, 72, 73}. Since effective root colonization is important for biocontrol, it is important to point out that *hcn* mutants are not impaired in root colonization⁴⁴. A recombinant strain of *P. putida* constructed to produce HCN shows better disease suppression ability than the parental strain in a system consisting of wheat seedling leaves infected by *Septoria tritici* or *Puccinia recondita* f. sp. tritici²⁵. In a collection of 29 strains of fluorescent pseudomonads examined, HCN production correlates positively with biocontrol ability²³. Thus, it appears that some plants can borrow cyanogenesis from selected bacteria, to defend themselves against pathogenic fungi. However, in another study, no relationship was found between the number of cyanogenic fluorescent pseudomonads and the degree of disease-suppressiveness of various Swiss soils⁵⁵. This illustrates well the fact that biocontrol of root diseases by fluorescent pseudomonads is multi-factorial and not mediated by a single dominant trait (see Chapter 15, Volume 1, by Moenne-Loccoz and Défago). In conclusion, under well-defined experimental conditions, cyanide can be shown to act as a biocontrol factor. However, from a bacterial perspective, a distinction between cyanide as a virulence factor and cyanide as a biocontrol factor is probably not critical.

6. CONCLUSIONS

In cyanogenic pseudomonads, HCN production is very strictly controlled at several levels. (a) At the transcriptional level, ANR and — in *P. aeruginosa* — the quorum sensing regulators LasR and RhIR act as positive control elements on the *hcnA* promoter. (b) At a post-transcriptional level, the GacS/GacA regulatory cascade controls the accessibility of the *hcnA* RBS with great efficacy. (c) At the protein level, HCN synthase is very sensitive to irreversible inactivation by high O₂ concentrations. Thus, if the idea were correct that HCN could serve as a chemical defense for bacteria in their ecological niches, then it is striking to note that the HCN producers use this strategy with utmost caution.

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REFERENCES

1. Aarons, S., Abbas, A., Adams, C., Fenton, A., and O'Gara, F., 2000, A regulatory RNA (PrrB RNA) modulates expression of secondary metabolite genes in *Pseudomonas fluorescens* F113. *J. Bacteriol.*, 182:3913–3919.
2. Achebach, S., Zeuner, Y., and Uden, G., 2003, Control of the O₂ sensor/regulator FNR of *Escherichia coli* by O₂ and reducing agents in vivo and in vitro. In P. Dürre and B. Friedrich (ed.), *Regulatory Networks in Prokaryotes*, pp. 93–99. Horizon Press, Norfolk, UK.
3. Askeland, R.A. and Morrison, S.M., 1983, Cyanide production by *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.*, 45:1802–1807.
4. Bakker, A.W. and Schippers, B., 1987, Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomonas* spp.-mediated plant growth-stimulation. *Soil Biol. Biochem.*, 19:451–457.
5. Bateman, A., Birney, E., Durbin, R., Eddy, S.R., Howe, K.L., and Sonnhammer, E.L., 2000, The Pfam protein families databases. *Nucleic Acids Res.*, 28:263–266.
6. Belyaeva, T.A., Rhodius, V.A., Webster, C.L., and Busby, S.J.W., 1998, Transcription activation at promoters carrying tandem DNA sites for the *Escherichia coli* cyclic AMP receptor protein: Organisation of the RNA polymerase α subunits. *J. Mol. Biol.*, 277:789–804.
7. Blumer, C. and Haas, D., 2000, Mechanism, regulation, and ecological role of bacterial cyanide biosynthesis. *Arch. Microbiol.*, 173:170–177.
8. Blumer, C. and Haas, D., 2000, Iron regulation of the *hcnABC* genes encoding hydrogen cyanide synthase depends on the anaerobic regulator ANR rather than on the global activator GacA in *Pseudomonas fluorescens* CHA0. *Microbiology*, 146:2417–2424.
9. Blumer, C., Heeb, S., Pessi, G., and Haas, D., 1999, Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites. *Proc. Natl. Acad. Sci. USA*, 96:14073–14078.
10. Castric, P.A., 1975, Hydrogen cyanide, a secondary metabolite of *Pseudomonas aeruginosa*. *Can. J. Microbiol.*, 21:613–618.
11. Castric, P.A., 1977, Glycine metabolism by *Pseudomonas aeruginosa*: Hydrogen cyanide biosynthesis. *J. Bacteriol.*, 130:826–831.
12. Castric, P.A., 1981, The metabolism of hydrogen cyanide by bacteria. In B. Vennesland, E.E. Conn, C.J. Knowles, J. Westley, and F. Wissing (eds), *Cyanide in biology*, pp. 233–261. Academic Press, London.
13. Castric, P.A., 1983, Hydrogen cyanide production by *Pseudomonas aeruginosa* at reduced oxygen levels. *Can. J. Microbiol.*, 29:1344–1349.
14. Castric, P.A., 1994, Influence of oxygen on the *Pseudomonas aeruginosa* hydrogen cyanide synthase. *Curr. Microbiol.*, 29:19–21.
15. Castric, P.A., Ebert, R.F., and Castric, K.F., 1979, The relationship between growth phase and cyanogenesis in *Pseudomonas aeruginosa*. *Curr. Microbiol.*, 2:287–292.
16. Castric, P.A., Castric, K.F., and Meganathan, R., 1981, Factors influencing the termination of cyanogenesis in *Pseudomonas aeruginosa*. In B. Vennesland, E.E. Conn, C.J. Knowles, J. Westley, and F. Wissing (eds), *Cyanide in biology*, pp. 263–274. Academic Press, London.

17. Chancey, S.T., Wood, D.W., Pierson, E.A., and Pierson, L.S., III, 2002, Survival of GacS/GacA mutants of the biological control bacterium *Pseudomonas aureofaciens* 30-84 in the wheat rhizosphere. *Appl. Environ. Microbiol.*, 68:3308–3314.
18. Clawson, B.J. and Young, C.C., 1913, Preliminary report on the production of hydrocyanic acid by bacteria. *J. Biol. Chem.*, 15:419–422.
19. Comolli, J.C. and Donohue, T.J., 2002, *Pseudomonas aeruginosa* RoxR, a response regulator related to *Rhodobacter sphaeroides* PrrA, activates expression of the cyanide-insensitive terminal oxidase. *Mol. Microbiol.*, 45:755–768.
20. Cooper, M., Tavankar, G.R., and Williams, H.D., 2003, Regulation of expression of the cyanide insensitive terminal oxidase in *Pseudomonas aeruginosa*. *Microbiology*, 149:1275–1284.
21. Cunningham, L., Pitt, M., and Williams, H.D., 1997, The *cioAB* genes from *Pseudomonas aeruginosa* code for a novel cyanide-insensitive terminal oxidase related to the cytochrome *bd* quinol oxidases. *Mol. Microbiol.*, 24:579–591.
22. De Bellis, P. and Ercolani, G.L., 2001, Growth interactions during bacterial colonization of seedling rootlets. *Appl. Environ. Microbiol.*, 67:1945–1948.
23. Ellis, R.J., Timms-Wilson, T.M., and Bailey, M.J., 2000, Identification of conserved traits in fluorescent pseudomonads with antifungal activity. *Environ. Microbiol.*, 2:274–284.
24. Falk-Krzesinski, H.J. and Wolfe, A.J., 1998, Genetic analysis of the *nuo* locus, which encodes the proton-translocating NADH dehydrogenase in *Escherichia coli*. *J. Bacteriol.*, 180:1174–1184.
25. Flaishman, M.A., Eyal, Z., Zilberstein, A., Voisard, C., and Haas, D., 1996, Suppression of *Septoria tritici* blotch and leaf rust of wheat by recombinant cyanide-producing strains of *Pseudomonas putida*. *Mol. Plant Microbe Interact.*, 9:642–645.
26. Firoved, A.M. and Deretic, V., 2003, Microarray analysis of global gene expression in mucoid *Pseudomonas aeruginosa*. *J. Bacteriol.*, 185:1071–1081.
27. Friedheim, E.A.H., 1934, The effect of pyocyanine on the respiration of some normal tissues and tumours. *Biochem. J.*, 28:173–179.
28. Gallagher, L.A. and Manoil, C., 2001, *Pseudomonas aeruginosa* PAO1 kills *Caenorhabditis elegans* by cyanide poisoning. *J. Bacteriol.*, 183:6207–6214.
29. Gallagher, L., McKnight, S.L., Kuznetsova, M.S., Pesci, E.C., and Manoil, C., 2002, Functions required for extracellular quinolone signaling by *Pseudomonas aeruginosa*. *J. Bacteriol.*, 184:6472–6480.
30. Goldfarb, W.B. and Margraf, H., 1967, Cyanide production by *Pseudomonas aeruginosa*. *Ann. Surg.*, 165:104–110.
31. Haas, D., Keel, C., and Reimann, C., 2002, Signal transduction in plant-beneficial rhizobacteria with biocontrol properties. *Antonie van Leeuwenhoek*, 81:385–395.
32. Hafner, E.W. and Wellner, D., 1979, Reactivity of the imino acids formed in the amino acid oxidase reaction. *Biochemistry*, 18:411–417.
33. Heeb, S., Blumer, C., and Haas, D., 2002, Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct formation in *Pseudomonas fluorescens* CHA0. *J. Bacteriol.*, 184:1046–1056.
34. Højberg, O., Schnider, U., Winteler, H.V., Sørensen, J., and Haas, D., 1999, Oxygen-sensing reporter strain of *Pseudomonas fluorescens* for monitoring the distribution of low-oxygen habitats in soil. *Appl. Environ. Microbiol.*, 65:4085–4093.
35. Jones, D.A., 1998, Why are so many food plants cyanogenic? *Phytochemistry*, 47:155–162.
36. Jones, D.T., Taylor, W.R., and Thornton, J.M., 1994, A model recognition approach to the prediction of all-helical membrane protein structure and topology. *Biochemistry*, 33:3038–3049.
37. Klein, P., Kanehisa, M., and DeLisi, C., 1985, The detection and classification of membrane-spanning proteins. *Biochem. Biophys. Acta*, 815:468–476.

38. Knowles, C.J. and Bunch, A.W., 1986, Microbial cyanide metabolism. *Adv. Microb. Physiol.*, 27:73–111.
39. Kralik, C.A. and Castric, P.A., 1979, Respiration and cyanogenesis in *Pseudomonas aeruginosa*. *Curr. Microbiol.*, 3:71–74.
40. Kremer, R.J. and Souissi, T., 2001, Cyanide production by rhizobacteria and potential for suppression of weed seedling growth. *Curr. Microbiol.*, 43:182–186.
41. Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E.L., 2001, Predicting transmembrane topology with a hidden Markov model. Application to complete genomes. *J. Mol. Biol.*, 305:567–580.
42. Kunz, D.A., Chen, J.L., and Pan, G., 1998, Accumulation of α -keto acids as essential components in cyanide assimilation by *Pseudomonas fluorescens* NCIMB 11764. *Appl. Environ. Microbiol.*, 64:4452–4459.
43. Laville, J., Voisard, C., Keel, C., Maurhofer, M., Défago, G., and Haas, D., 1992, Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. *Proc. Natl. Acad. Sci. USA*, 89:1562–1566.
44. Laville, J., Blumer, C., Von Schroetter, C., Gaia, V., Défago, G., Keel, C., and Haas, D., 1998, Characterization of the *hcnABC* gene cluster encoding hydrogen cyanide synthase and anaerobic regulation by ANR in the strictly aerobic biocontrol agent *Pseudomonas fluorescens* CHA0. *J. Bacteriol.*, 180:3187–3196.
45. Lorck, H., 1948, Production of hydrocyanic acid by bacteria. *Physiol. Plant.*, 1:142–146.
46. Malkin, R. and Rabinowitz, J.C., 1967, The reactivity of clostridial ferredoxin with iron chelating agents and 5,5'-dithiobis-2-nitro-benzoic acid. *Biochemistry*, 6:3880–3891.
47. Matsushita, K., Yamada, M., Shinagawa, E., Adachi, O., and Ameyama, M., 1983, Membrane-bound respiratory chain of *Pseudomonas aeruginosa* grown aerobically. A KCN-insensitive alternate oxidase chain and its energetics. *J. Biochem.*, 93:1137–1144.
48. Nishiya, Y. and Imanaka, T., 1998, Purification and characterization of a novel glycine oxidase from *Bacillus subtilis*. *FEBS Lett.*, 438:263–266.
49. Olsiewski, P.J., Kaczorowski, G.J., and Walsh, C., 1980, Purification and properties of D-amino acid dehydrogenase, an inducible membrane-bound iron-sulfur flavoenzyme from *Escherichia coli* B. *J. Biol. Chem.*, 255:4487–4494.
50. Pesci, E.C., Milbank, J.B.J., Pearson, J.P., McKnight, S., Kende, A.S., Greenberg, E.P., and Iglewski, B.H., 1999, Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA*, 96:11229–11234.
51. Pessi, G. and Haas, D., 2000, Transcriptional control of the hydrogen cyanide biosynthetic genes *hcnABC* by the anaerobic regulator ANR and the quorum-sensing regulators LasR and RhlR in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 182:6940–6949.
52. Pessi, G. and Haas, D., 2001, Dual control of hydrogen cyanide biosynthesis by the global activator GacA in *Pseudomonas aeruginosa* PAO1. *FEMS Microbiol. Lett.*, 200:73–78.
53. Pessi, G., Williams, F., Hindle, Z., Heurlier, K., Holden, M.T.G., Camara, M., Haas, D., and Williams, P., 2001, The global posttranscriptional regulator RsmA modulates production of virulence determinants and N-acylhomoserine lactones in *Pseudomonas aeruginosa*. *J. Bacteriol.*, 183:6676–6683.
54. Ramette, A., Frapolli, M., Défago, G., and Moënne-Loccoz, Y., 2003, Phylogeny of HCN synthase-encoding *hcnBC* genes in biocontrol fluorescent pseudomonads and relationship with host plant species and HCN synthesis ability. *Mol. Plant Microbe Interact.*, 16:525–535.
55. Ramette, A., Moënne-Loccoz, Y., and Défago, G., 2003, Prevalence of fluorescent pseudomonads producing antifungal phloroglucinols and/or hydrogen cyanide in soils naturally suppressive or conducive to tobacco black root rot. *FEMS Microbiol. Ecol.*, 44:35–43.
56. Rao, M.J.K. and Argos, P., 1986, A conformational preference parameter to predict helices in integral membrane proteins. *Biochim. Biophys. Acta*, 869:197–214.

57. Raybuck, S.A., 1992, Microbes and microbial enzymes for cyanide degradation. *Biodegradation*, 3:3–18.
58. Reimmann, C., Beyeler, M., Latifi, A., Winteler, H., Foglino, M., Lazdunski, A., and Haas, D., 1997, The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer *N*-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. *Mol. Microbiol.*, 24:309–319.
59. Reissmann, S., Hochleitner, E., Wang, H., Paschos, A., Lottspeich, F., Glass, R.S., and Böck, A., 2003, Taming of a poison: Biosynthesis of the NiFe-hydrogenase cyanide ligands. *Science*, 299:1067–1070.
60. Ryan, R.W. and Tilton, R.C., 1977, The isolation of rhodanese from *Pseudomonas aeruginosa* by affinity chromatography. *J. Gen. Microbiol.*, 103:197–199.
61. Sabra, W., Kim, E.-J., and Zeng, A.-P., 2002, Physiological responses of *Pseudomonas aeruginosa* PAO1 to oxidative stress in controlled microaerobic and aerobic cultures. *Microbiology*, 148:3195–3202.
62. Sánchez-Contreras, M., Martín, M., Villaceros, M., O’Gara, F., Bonilla, I., and Rivilla, R., 2002, Phenotypic selection and phase variation occur during alfalfa root colonization by *Pseudomonas fluorescens* F113. *J. Bacteriol.*, 184:1587–1596.
63. Sawers, R.G., 1991, Identification and molecular characterization of a transcriptional regulator from *Pseudomonas aeruginosa* PAO1 exhibiting structural and functional similarity to the FNR protein of *Escherichia coli*. *Mol. Microbiol.*, 5:1469–1481.
64. Settembre, E.C., Dorrestein, P.C., Park, J.-H., Augustine, A.M., Begley, T.P., and Ealick, S.E., 2003, Structural and mechanistic studies on ThiO, a glycine oxidase essential for thiamin biosynthesis in *Bacillus subtilis*. *Biochemistry*, 42:2971–2981.
65. Solomonson, L.P., 1981, Cyanide as a metabolic inhibitor. In B. Vennesland, E.E. Conn, C.J. Knowles, J. Westley and F. Wissing (eds), *Cyanide in Biology*, pp. 11–28. Academic Press, London.
66. Tatusov, R.L., Natale, D.A., Garkavtsev, I.V., Tatusova, T.A., Shankavaram, U.T., Rao, B.S., Kiryutin, B., Galperin, M.Y., Fedorova, N.D., and Koonin, E.V., 2001, The COG database: New developments in phylogenetic classification of proteins from complete genomes. *Nucleic Acids Res.*, 29:22–28.
67. Tavankar, G.R., Mossialos, D., and Williams, H.D., 2003, Mutation or overexpression of a terminal oxidase leads to a cell division defect and multiple antibiotic sensitivity in *Pseudomonas aeruginosa*. *J. Biol. Chem.*, 278:4524–4530.
68. Trutko, S.M., Golovchenko, N.P., and Akimenko, V.K., 1979, Study of cyanide-resistant respiration of *Pseudomonas aeruginosa*. *Biokhimiya*, 44:720–728.
69. Valverde, C., Heeb, S., Keel, C., and Haas, D., 2003, RsmY, a small regulatory RNA, is required in concert with RsmZ for GacA-dependent expression of biocontrol traits in *Pseudomonas fluorescens* CHA0. *Mol. Microbiol.*, 50:1361–1379.
70. Villaceros, M., Power, B., Sánchez-Contreras, M., Lloret, J., Oruezabal, R.I., Martín, M., Fernández-Piñas, F., Bonilla, I., Whelan, C., Dowling, D.N., and Rivilla, R., 2003, Colonization behaviour of *Pseudomonas fluorescens* and *Sinorhizobium meliloti* in the alfalfa (*Medicago sativa*) rhizosphere. *Plant Soil*, 251:47–54.
71. Vennesland, B., Pistorius, E.K., and Gewitz, H.S., 1981, HCN production by microalgae. In B. Vennesland, E.E. Conn, C.J. Knowles, J. Westley, and F. Wissing (eds), *Cyanide in Biology*, pp. 349–362. Academic Press, London.
72. Voisard, C., Keel, C., Haas, D., and Défago, G., 1989, Cyanide production by *Pseudomonas fluorescens* helps suppress black root rot of tobacco under gnotobiotic conditions. *EMBO J.*, 8:351–358.
73. Voisard, C., Bull, C.T., Keel, C., Laville, J., Maurhofer, M., Schnider, U., Défago, G., and Haas, D., 1994, Biocontrol of root diseases by *Pseudomonas fluorescens* CHA0: Current

- concepts and experimental approaches. In F. O'Gara, D.N. Dowling, and B. Boesten (eds), *Molecular Ecology of Rhizosphere Microorganisms*, pp. 67–89. VCH, Weinheim.
74. Von Tigerstrom, M. and Campbell, J.J.R., 1966, The accumulation of α -ketoglutarate by suspensions of *Pseudomonas aeruginosa*. *Can. J. Microbiol.*, 12:1005–1013.
 75. Whiteley, M., Lee, K.M., and Greenberg, E.P., 1999, Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA*, 96:13904–13909.
 76. Wierenga, R.K., Terpstra, P., and Hol, W.G., 1986, Prediction of the occurrence of the ADP-binding $\beta\alpha\beta$ -fold in proteins, using an amino acid sequence fingerprint. *J. Mol. Biol.*, 187:101–107.
 77. Winson, M.K., Camara, M., Latifi, A., Foglino, M., Chhabra, S.R., Daykin, M., Bally, M., Chapon, V., Salmond, G.P.C., Bycroft, B.W., Lazdunski, A., Stewart, G.S.A.B., and Williams, P., 1995, Multiple *N*-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA*, 92:9427–9431.
 78. Winteler, H.V. and Haas, D., 1996, The homologous regulators ANR of *Pseudomonas aeruginosa* and FNR of *Escherichia coli* have overlapping but distinct specificities for anaerobically inducible promoters. *Microbiology*, 142:685–693.
 79. Wissing, F., 1974, Cyanide formation from oxidation of glycine by a *Pseudomonas* species. *J. Bacteriol.*, 117:1289–1294.
 80. Wissing, F., 1975, Cyanide production from glycine by a homogenate from a *Pseudomonas* species. *J. Bacteriol.*, 121:695–699.
 81. Wissing, F., 1983, Anaerobic column chromatography in the presence of detergents and its application to a bacterial HCN-producing enzyme. *J. Microbiol. Methods*, 1:31–39.
 82. Wissing, F. and Andersen, K.A., 1981, The enzymology of cyanide production from glycine by a *Pseudomonas* species. In B. Vennesland, E.E. Conn, C.J. Knowles, J. Westley, and F. Wissing (eds), *Cyanide in Biology*, pp. 275–288. Academic Press, London.
 83. Zanker, H., Lurz, G., Langridge, U., Langridge, P., Kreusch, D., and Schröder, J., 1994, Octopine and nopaline oxidases from Ti plasmids of *Agrobacterium tumefaciens*: Molecular analysis, relationship, and functional characterization. *J. Bacteriol.*, 176:4511–4517.
 84. Zimmermann, A., Reimann, C., Galimand, M., and Haas, D., 1991, Anaerobic growth and cyanide synthesis of *Pseudomonas aeruginosa* depend on *anr*, a regulatory gene homologous with *fnr* of *Escherichia coli*. *Mol. Microbiol.*, 5:1483–1490.
 85. Zuber, S., Carruthers, F., Keel, C., Mattart, A., Blumer, C., Pessi, G., Gigot-Bonnefoy, C., Schnider-Keel, U., Heeb, S., Reimann, C., and Haas, D., 2003, GacS sensor domains pertinent to the regulation of exoproduct formation and to the biocontrol potential of *Pseudomonas fluorescens* CHA0. *Mol. Plant Microbe Interact.*, 16:634–644.

POLYKETIDE ANTIBIOTICS OF *PSEUDOMONAS*

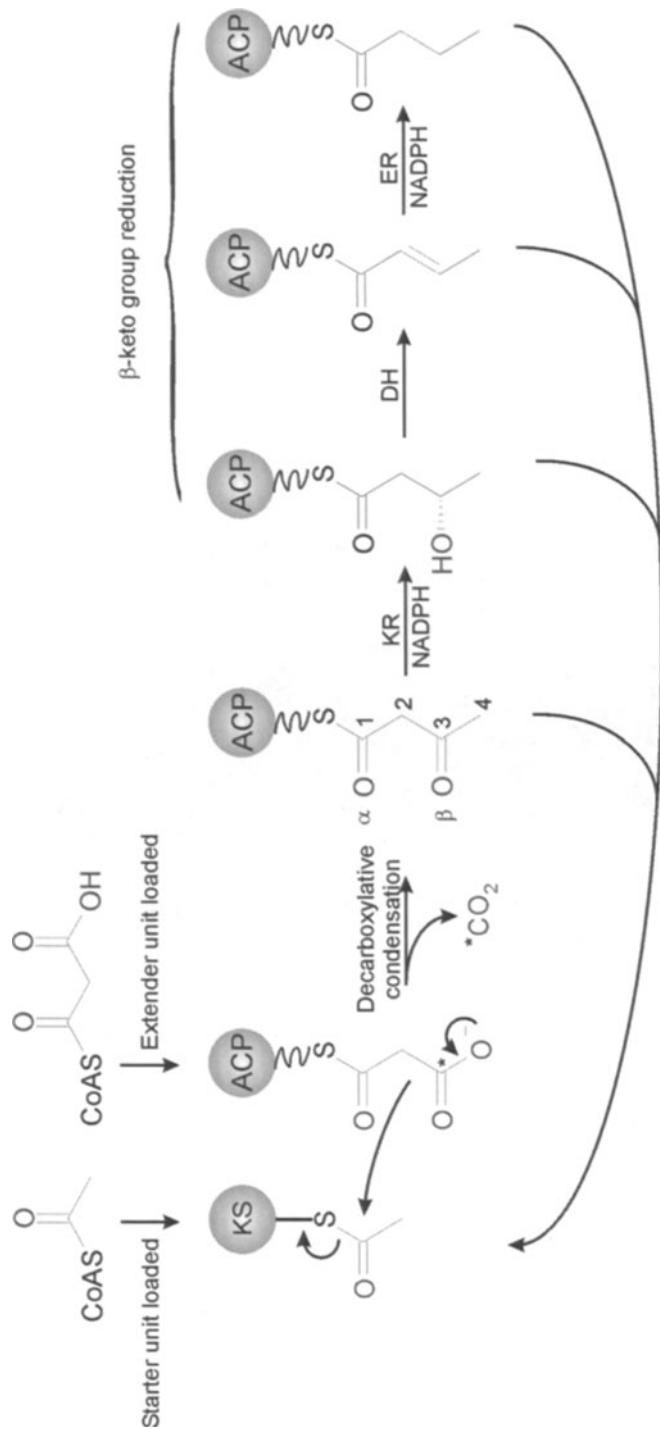
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1. INTRODUCTION

Polyketides are diverse natural products built from the successive decarboxylative condensative additions of small extender units from acids like malonate and methylmalonate to a starter unit (Figure 1). Without further processing, this would generate a keto group at carbon 3, 5, 7 etc., hence the name polyketide. Diversity can be generated not only by the choice of starter and extender units and number of condensation cycles, but also by the extent of processing, reduction and dehydration, at the β -keto group of the carbon chain. Additional tailoring enzymes may be involved in functional group modifications, and another level of complexity may come with patterns of cyclisation and stereochemical variations. This generates a diversity of structure and function with a wealth of important pharmacological activities including antibacterial, antifungal, antiparasitic, anti-cancer, anti-cholesterolemic and immunosuppressive properties. It is the nature of their biosynthesis that enables this complexity.

Polyketide biosynthesis is catalysed by polyketide synthases (PKS). If the catalytic sites of PKSs are encoded by domains within large multifunctional proteins, then they are classified as Type I PKSs. The domains of Type I PKSs each encode a distinct step and may be grouped in modules, with each module



Growing polyketide chain transferred back to KS or onto next KS for subsequent extension and possible reduction

Figure 1. Polyketide biosynthesis. Ketosynthase (KS), acyl carrier protein (ACP), ketoreductase (KR), dehydratase (DH), enoyl reductase (ER).

encoding a specific round of condensation and reduction²⁷. Type I products are typically complex, fairly reduced structures, as exemplified by erythromycin, rapamycin, avermectin and tylosin^{8, 9, 27, 64, 97}. Fungal PKSs have a Type I architecture with active sites on a single multifunctional protein. However, instead of having separate modules for each round of condensation and reduction, these fungal PKSs can use domains iteratively. That is, at least some of the enzymes are used repeatedly in successive rounds of chain extension and reduction. Examples of fungal Type I iterative PKSs include 6-methylsalicylic acid and lovastatin PKSs^{10, 45}.

A second class of PKSs exist which are monofunctional proteins (Type II) that often associate in a complex. That is, the catalytic sites required for polyketide synthesis are carried on discrete proteins. Type II PKS products are typically phenolic aromatic polyketides, for example, actinorhodin, tetracenomycin, oxytetracycline and daunorubicin^{14, 65, 75, 104}. One or more of the enzymes in Type II PKSs may also be used iteratively.

Polyketide biosynthesis for these Type I and Type II PKSs systems occurs by a process similar to fatty acid biosynthesis (Figure 1). It initiates with a simple carboxylic acid starter unit, typically acetate or propionate, which is activated as a CoA thioester and transferred, possibly by an acyl-transferase (AT), to the cysteine group of a β -ketoacyl synthase (KS). A dicarboxylic acid extender unit, usually a malonate or methyl malonate-CoA thioester, is transferred to the thiol group of the phosphopantetheine arm of an acyl carrier protein (ACP). The starter unit is added to the extender unit by a decarboxylative condensation catalysed by KS, so the diketide remains covalently attached to ACP as a thioester. Depending on the type of system, the β -keto group of the resulting intermediate may be left unreduced or partially or fully reduced by the sequential action of β -ketoacyl reductases (KR), dehydratases (DH) and enoyl reductases (ER). The growing chain is transferred from the ACP back to a KS and further rounds of elongation and possible reduction occur to achieve the full-length polyketide chain, which may be further modified by the activity of tailoring enzymes. The completed chain is typically released from the PKS by hydrolysis catalysed by a terminal thioesterase (TE) and may be cyclised and aromatised.

A third class of PKSs is the Type III PKSs that are typically found in higher plants⁹⁶, but which have also been found in Gram-positive bacteria³⁷ and fluorescent *Pseudomonads*⁷. The Type III plant chalcone and stilbene synthases consist of a single dimeric protein, which is capable of catalysing all of the different PKS reactions of decarboxylation, condensation, cyclisation and aromatisation from a single active site cavity³⁴. Unlike the Type I and II PKSs, the Type III PKSs lack an ACP and are not phosphopantetheinylated. Thus, they utilise acyl-CoA esters directly as a source of extender units instead of acyl-ACP thioesters.

Non-ribosomal peptide synthases (NRPS) are often associated with PKSs. These NRPSs are similar to Type I modular PKSs in their architectural organisation¹⁰². However, they encode the activation and incorporation of amino acids, catalysed by an adenylation domain (A), which generates aminoacyl-AMP and tethers it to a peptidyl carrier protein (PCP) as a thioester via a phosphopantetheine arm. The condensation domain (C) catalyses the joining of this aminoacyl-S-PCP with a downstream aminoacyl-S-PCP to generate a peptide bond. As with PKS, successive rounds of elongation occur along with optional modifications such as epimerisation, methyl transferase and heterocyclisation. The full-length peptide chain is released from the PCP by the hydrolytic activity of a TE. An example of a pharmacologically important non-ribosomal peptide is antibacterial glycopeptide vancomycin, which inhibits cell wall biosynthesis. Natural products have been identified that are synthesised from clusters containing both PKSs and NRPSs, which will produce complex structures derived from both carboxylic and amino acids. For example the NRPS may generate an unusual amino acid starter unit that can then be extended by a PKS, as is the case for pyoluteorin biosynthesis^{76, 106}.

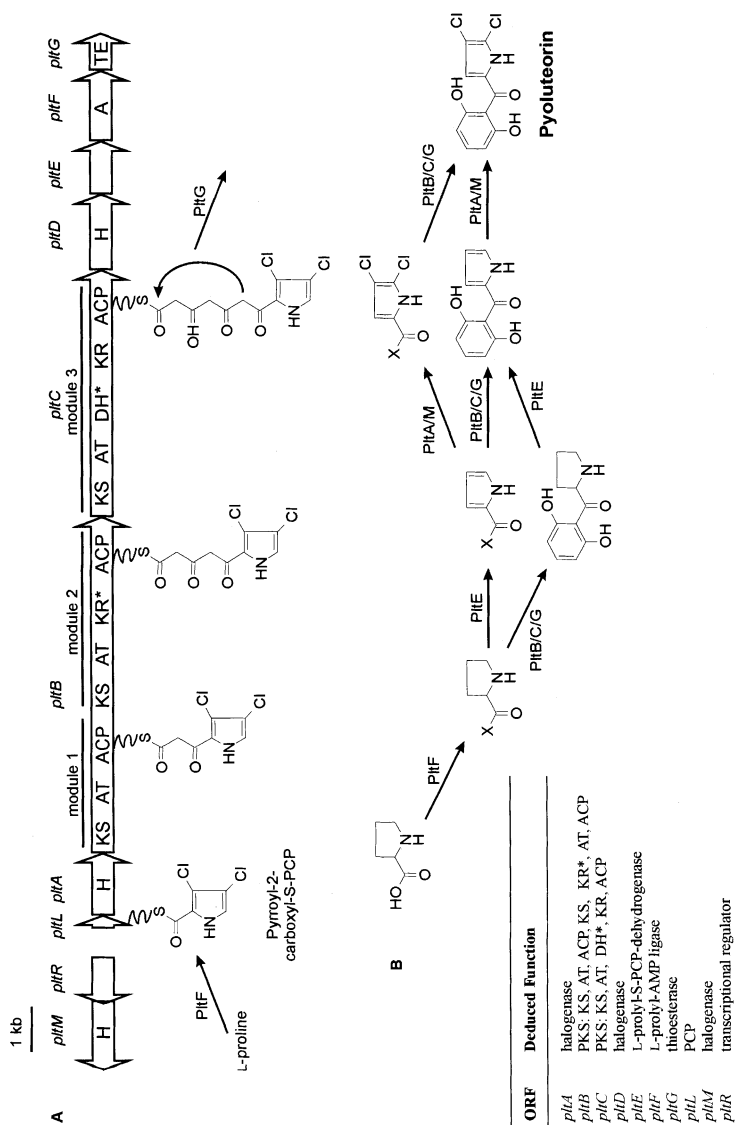
This chapter will focus on five polyketides that have been well characterised in *Pseudomonads*. These include the phytotoxin coronatine, antifungals pyoluteorin, 2,4-diacetylphloroglucinol (DAPG), antibacterial mupirocin and the anti-tumour pederin. Interestingly their biosynthesis pathways show examples of Type I, II, III PKSs and NRPS organisation.

2. PYOLUTEORIN

2.1. Pyoluteorin Activity and Structure

Pyoluteorin is an antifungal metabolite produced by various *Pseudomonads*, such as the biocontrol agent *Pseudomonas fluorescens* Pf-5⁴⁶. In particular, pyoluteorin inhibits Oomycete fungi, including the phytopathogen *Pythium ultimum*, which infects many plants. *P. fluorescens* inhabits the rhizosphere and suppresses plant diseases such as seedling damping-off and root rot caused by seed and root infection by *P. ultimum*⁴⁶.

Pyoluteorin is a chlorinated aromatic polyketide consisting of a resorcinol ring derived from polyketide biosynthesis attached to a bichlorinated pyrrole ring^{25, 78} (Figure 2). ¹³C-labelled proline feeding experiments revealed that L-proline is the primary precursor to the pyrrole moiety of pyoluteorin⁷⁶.



2.2. Pyoluteorin Biosynthetic Cluster

Tn5 transposon mutagenesis and complementation of a Plt^- strain identified a 21 kb region responsible for pyoluteorin biosynthesis⁵⁸. Sequence analysis of 24 kb encompassing this region revealed 10 open reading frames (ORFs)^{76, 78} (Figure 2A and B) (Accession no. AF081920). Two ORFs *pltB* and *pltC*, which show sequence similarity with Type I PKS are believed to catalyse the formation of the resorcinol ring. *PltB* contains two modules that would extend a starter unit by two acetate units from malonyl-CoA extender units. *PltC* contains one module that would then extend this intermediate by an acetate unit from another malonyl-CoA extender unit. Interestingly *PltB* does not contain an obvious loading domain and *PltC* does not contain a terminal TE domain typical of, but not absolutely required by Type I PKS. Both are also predicted to contain an inactive domain. *PltB* contains a KR domain which lacks the NADP(H) binding region required for catalytic activity, and *PltC* contains a DH domain, with limited sequence similarity to other DHs, that lacks the active site motif⁷⁸.

pltE, *pltF* and *pltL* are thought to be involved in the activation of a proline derivative and generation of the pyrrole ring via a NRPS thiotemplate mechanism¹⁰⁶. *PltF* shows similarity to amino acid activating enzymes. In such NRPS systems, an amino acid is activated to an amino acyl-AMP intermediate, which is subsequently covalently attached by a thioester to a phosphopantetheinyl arm attached to a peptidyl carrier protein (PCP). *PltL* shows weak similarity to such PCP domains¹⁰⁶, and *PltE* to acyl-CoA dehydrogenases⁷⁶.

pltA, *pltD* and *pltM* are related to FADH₂-dependent halogenases which chlorinate secondary metabolites¹¹¹ and therefore may chlorinate the pyrrole ring. *PltA* and *PltM*, but not *PltD*, both contain an NADH cofactor binding motif. This suggests that *PltD* is inactive and may explain why there are three halogenases when the pyrrole moiety is only bichlorinated⁷⁶. The remaining ORFs are *pltG*, which is related to TEs, and *pltR*, which shows similarity to transcriptional activators of the LysR family⁹³.

2.3. Pyoluteorin Biosynthesis Model

A model for the biosynthesis of pyoluteorin from an L-proline-derived starter unit and 3-malonyl-CoA extender units catalysed by NRPS and PKS has been proposed, although the exact order of events has not yet been determined^{76, 106} (Figure 2A and B).

Thomas *et al.*¹⁰⁶ present biochemical evidence for the generation of a starter unit pyrrolyl-2-carboxylate by *PltF*, *PltE* and *PltL*. Initially L-proline would be activated to L-prolyl-AMP by the aminoadenylation domain of *PltF*, which would then be thioesterified to the PCP of *PltL* to generate L-prolyl-S-PCP.

The final pyrrole ring requires oxidation of this proline-derived carbon ring. Therefore, the bound intermediate is most likely catalysed by the L-prolyl-S-PCP-dehydrogenase PltE, followed by air oxidation to generate pyrrolyl-2-carboxyl-S-PCP¹⁰⁶. Tautomerisation and subsequent aromatisation of the pyrroline ring to the pyrrole could be a non-enzymatic process⁴.

The lack of an obvious loading domain within PltB suggests that the PltE/F/L NRPS complex may activate and then transfer a starter unit onto the first KS of module one of PltB. Alternatively, there may be an as yet unidentified transferase that is either inherent in the pathway or utilised from another pathway. The FADH₂-dependent halogenases PltA and PltM are most likely to catalyse chlorination of the pyrrole ring^{76, 111}. However, it is not known if the pyrrolyl-2-carboxyl-S-PCP intermediate is transferred to PltB before or after chlorination.

The transferred starter unit could then be extended by three malonyl-CoA extender units catalysed by the PKSs PltB and PltC. Formation of the resorcinol ring moiety requires dehydration at C3. However, as previously mentioned, functional DH domains have not been identified within PltB or PltC. Therefore, dehydration may be a non-enzymatic reaction that occurs after release of the bound intermediate from the PKS⁷⁸. Generation of pyoluteorin requires cyclisation of the resorcinol ring, which may occur simultaneously with thioester hydrolysis, perhaps catalysed by PltG (TE).

2.4. Regulation of Pyoluteorin Biosynthesis

The contribution of pyoluteorin to suppression of *P. ultimum* damping-off seems to differ depending on the host plant. In strains *P. fluorescens* Pf-5 and CHAO, pyoluteorin contributed minimally to suppression on cucumber, whereas it was required in Pf-5 for suppression on cotton^{46, 59}, and in strain CHAO for suppression on cress⁷⁰. Transcriptional fusion of an ice nucleation gene with the *plt* promoter region revealed that in Pf-5 expression of the pyoluteorin cluster was delayed on cucumber compared to cotton, and that pyoluteorin production was significantly affected by the carbon source, for example, it was lowered by glucose⁵⁸.

PltR shows similarity to transcriptional activators of the LysR family⁹³. Upstream of *pltR* is an inverted repeat that conforms to a LysR-type protein binding domain (an Ebright box motif) that is conserved within promoters activated by LysR-type proteins⁷⁶. Inactivation of *pltR* in Pf-5 resulted in loss of pyoluteorin production and reduced transcription of *pltB*, *pltE* and *pltF*, indicating that PltR is a positive transcriptional activator of pyoluteorin biosynthesis⁷⁶. However, the signals that induce LysR activation and the nature of a coinducer, which are typically required by LysR-type proteins, are not yet known.

Pyoluteorin biosynthesis is also modulated by the global regulators GacA, GacS and Lon, and multiple sigma factors: σ^S , the stationary sigma factor encoded by *rpoS*; σ^D , the housekeeping sigma factor encoded by *rpoD*; and possibly σ^H , the heat shock sigma factor. Amplification of *rpoD* increased pyoluteorin production in CHAO⁹⁴. GacA and GacS act together as a two component regulatory system required for the timely expression and accumulation of σ^S during transition from exponential to stationary phase¹¹⁶. GacS is a sensor protein that responds to the environment by autophosphorylation followed by transfer of the phosphate to its cognate response regulator GacA. GacA contains a DNA binding motif that would allow it to regulate gene expression by binding to the target gene promoter. Inactivation of GacA and GacS in Pf-5 abolished pyoluteorin production, but paradoxically inactivation of *rpoS* resulted in increased production suggesting that RpoS must be maintained at a critical level, neither too high nor too low¹¹⁷. Lon shows similarity to ATP-dependent serine proteases, which in *Escherichia coli* are heat shock proteins that non-specifically degrade denatured or non-functional intracellular proteins⁴⁰. Upstream of *lon* is a σ^{32} -like promoter sequence, which is a homologue of σ^H , thus transcription of *lon* may require σ^H (see ref. [117]). Inactivation of *lon* resulted in overproduction of pyoluteorin suggesting a role for proteolysis in the control of biosynthesis¹¹⁷.

3. MUPIROCIN

3.1. Mupirocin Activity and Structure

Mupirocin is used to control *Staphylococcus aureus*, particularly methicillin-resistant *S. aureus*, when other antibiotics are ineffective. It competitively inhibits bacterial isoleucyl-tRNA synthase (IleRS)⁴⁷ by interaction with the amino acid and ATP binding sites of IleRS¹¹⁸. Formation of Ile.tRNA is thus blocked, which inhibits protein synthesis. Despite low affinity for mammalian IleRS⁴⁸, mupirocin can only be used topically because it is quickly inactivated in the serum by hydrolysis of the ester linkage between MA and 9-HA. Furthermore, 95% is serum bound resulting in poor bioavailability²¹. Resistance to mupirocin is also spreading due to a gene for a mupirocin-insensitive IleRS²³.

Mupirocin is a mixture of polyketide pseudomonic acids produced by *P. fluorescens* NCIMB 10586. Pseudomonic acid A (C₂₆H₄₄O₉), which accounts for 90% of the mixture, consists of a C₁₇ unit (monic acid, MA) thought to be derived from an unsaturated polyketide containing a pyran ring, and a C₉ saturated fatty acid (9-hydroxynonanoic acid, 9-HN)^{19, 20, 36} (Figure 3). Pseudomonic acid B (8%) has an additional hydroxyl group at C8 (see ref. [19],

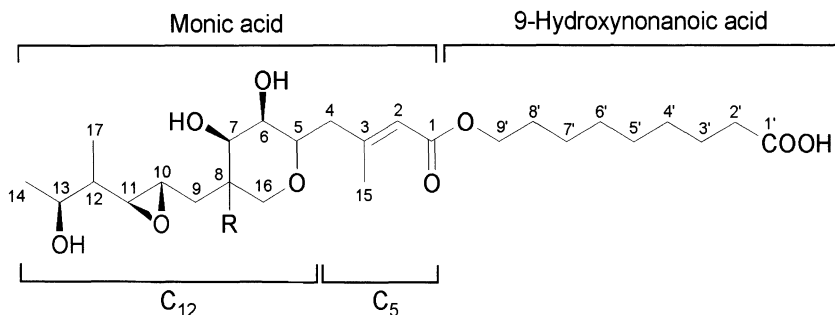


Figure 3. Structure of mupirocin (pseudomonic acid). Pseudomonic acid consists of a C_{17} unit (monic acid) derived from an unsaturated polyketide containing a pyran ring, and a C_9 saturated fatty acid (9-hydroxynonanoic acid). Pseudomonic acid A: $R = H$; pseudomonic acid B: $R = OH$; pseudomonic acid C: $R = H$, C10/C11 *E*-alkene; pseudomonic acid D: $R = H$, C4'/C5' *E*-alkene.

pseudomonic acid C (<2%) has a double bond in place of the epoxide group at C10–C11²², and pseudomonic acid D (<2%) has an unsaturated fatty acid side chain with an alkene group at C4'–C5'⁷⁹. Novel pseudomonic acid analogues have also been isolated from marine organisms, for example, *Altermonas* sp SANK 73390¹⁰¹.

Feeding experiments with isotopically labelled substrates showed that pseudomonic acid A is derived from a linear combination of acetate-derived units, except for C16 and C17 which are derived from the methyl of *S*-adenosylmethionine (SAM) and C15 which has been proposed to be derived from acetate via 3-hydroxy-3-methylglutarate (HMG)^{33, 66}. The oxygen atoms attached to C1, C5, C7, C13 and C9 are acetate-derived and the C1 ester linkage is proposed to be formed from condensation of MA and 9-HA subunits⁶⁷.

3.2. Mupirocin Biosynthetic Cluster

Mutational inactivation, gene cloning and reverse genetics identified a >65 kb region involved in mupirocin biosynthesis¹¹⁵. Sequence analysis of a 74 kb region (Accession no. AF318063) revealed 35 ORFs³⁰ (Figure 4). The cluster contains six multifunctional genes with the architecture of Type I PKSs (*mmpA*, *B*, *C*, *D*, *E* and *F*) and various individual genes (*mupE*, *F*, *J*, *K*, *Q*, *U* and *macpA*, *B*, *C*, *D*, *E*) with predicted Type I PKSs and fatty acid synthase functions, and four individual genes which show similarity to Type II systems (*mupB*, *mupD*, *mupG* and *mupS*). It therefore seems that the mupirocin cluster is a combination of both Type I and Type II PKS systems. The predicted functions of these orfs are shown in Figure 4.

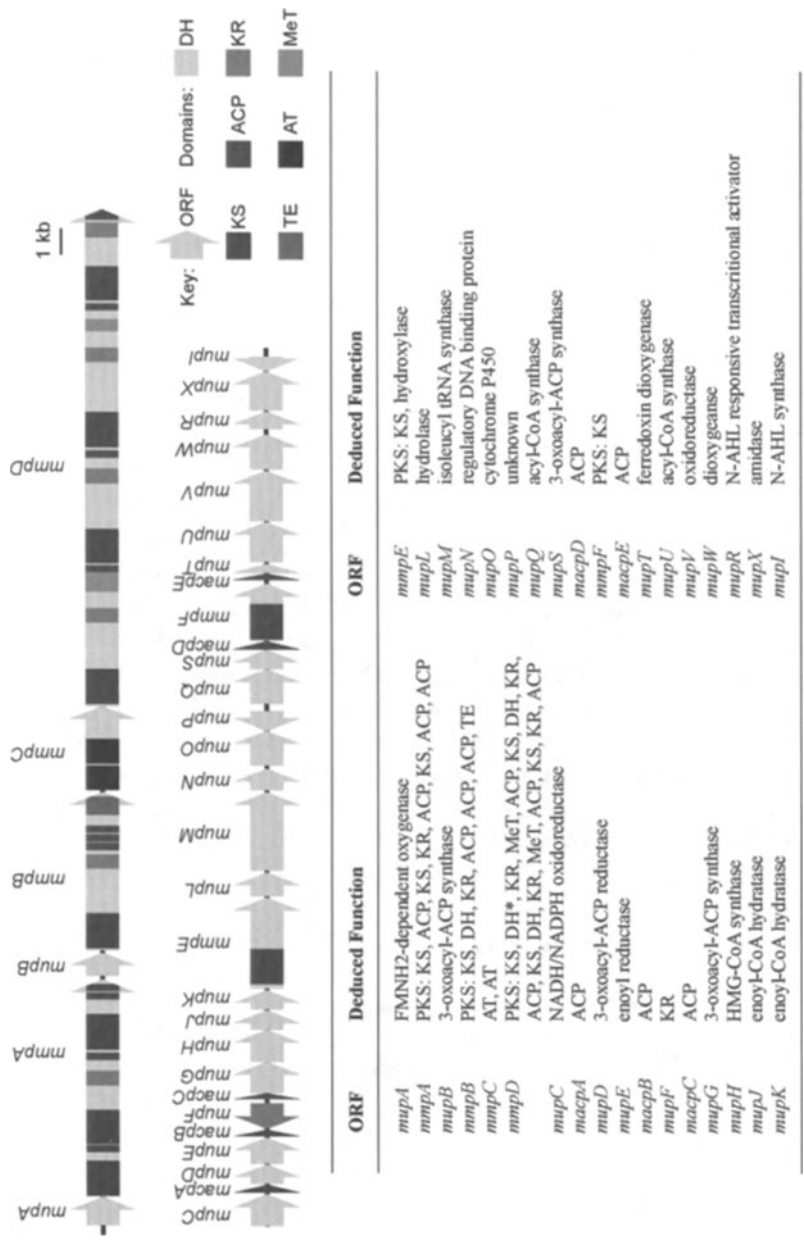


Figure 4. Organisation of the mupirocin biosynthesis cluster. Ketosynthase (KS), acyl carrier protein (ACP), ketoreductase (KR), dehydratase (DH), thioesterase (TE), acyltransferase (AT), methyl transferase (MeT), 3-hydroxy-3-methylglutaric acid (HMG), N-acetyl homoserine lactone (N-AHL), * indicates an inactive domain.

Upstream of the putative *mupA* promoter region are genes for tRNA_{Val} and tRNA_{Asp}. Southern blotting and sequence comparisons with contiguous sequence from the ongoing *P. fluorescens* SBW25 genome sequencing project (www.sanger.ac.uk/Projects/P_fluorescens/) revealed that *mup* DNA was not present in *P. fluorescens* SBW25, which does not produce mupirocin, but DNA on the other side of the tRNA cluster was present in SBW25. This suggests that the *mup* cluster may be an insertion integrated by recombination at tRNA genes as observed for a number of phage and mobile elements⁴⁴. Furthermore, MupM showed significant similarity with several mupirocin-resistant IleRS and conferred mupirocin resistance on *E. coli*³⁰. The *mup* gene cluster may therefore be a phenotypic island that could carry mupirocin biosynthesis and resistance from one strain to another.

There seem to be some notable features that are unusual but not unique features of the *mup* pathway. First, there is a lack of obvious linear relationship between gene order and biosynthetic pathway. Second, there are only two AT domains, which are separate from the Type I modules and presumably act *in trans*. Monofunctional ATs have been identified within the pederin biosynthetic cluster described in Section 4⁸⁶. Third, there are a tandem ACP doublet (ACP3 and 4) and a tandem ACP triplet (ACP5, 6 and 7). These may hold multiple substrates prior to condensation or allow increased throughput at rate-limiting steps in the pathway. Tandem ACPs have also been observed in various Type I iterative fungal PKS, for example, WA, a naphthopyrone synthase of *Aspergillus nidulans* in which both ACPs function but only one is required³⁵. Finally, there is only a single TE despite two predicted products, MA and 9-HN.

3.3. Mupirocin Biosynthesis Model

A model for the mupirocin biosynthetic pathway has been proposed which is largely supported by the sequence³⁰. It has been demonstrated that mupirocin is derived mainly from acetate and proposed that separate PKS and FASs are involved in its assembly from MA and 9-HA via an esterification^{19, 67}. Based on other PKS/FAS systems, a plausible biosynthetic pathway for MA is proposed in Figure 5A.

The *mup* cluster lacks an obvious loading domain, which should catalyse the covalent attachment of an activated starter unit to the first KS domain of a PKS. No AT domains were identified within the condensation modules themselves so the AT domains of *mmpC* may perform the task of loading and transferring the growing polyketide chain to each KS or other unidentified transferase activities may be involved.

It has been proposed that biosynthesis of MA starts with the production of the C₁₂ pentaketide moiety (**4**, from Figure 5A) by the PKS MmpD, which then acts as a primer for biosynthesis of the remainder of the MA polyketide

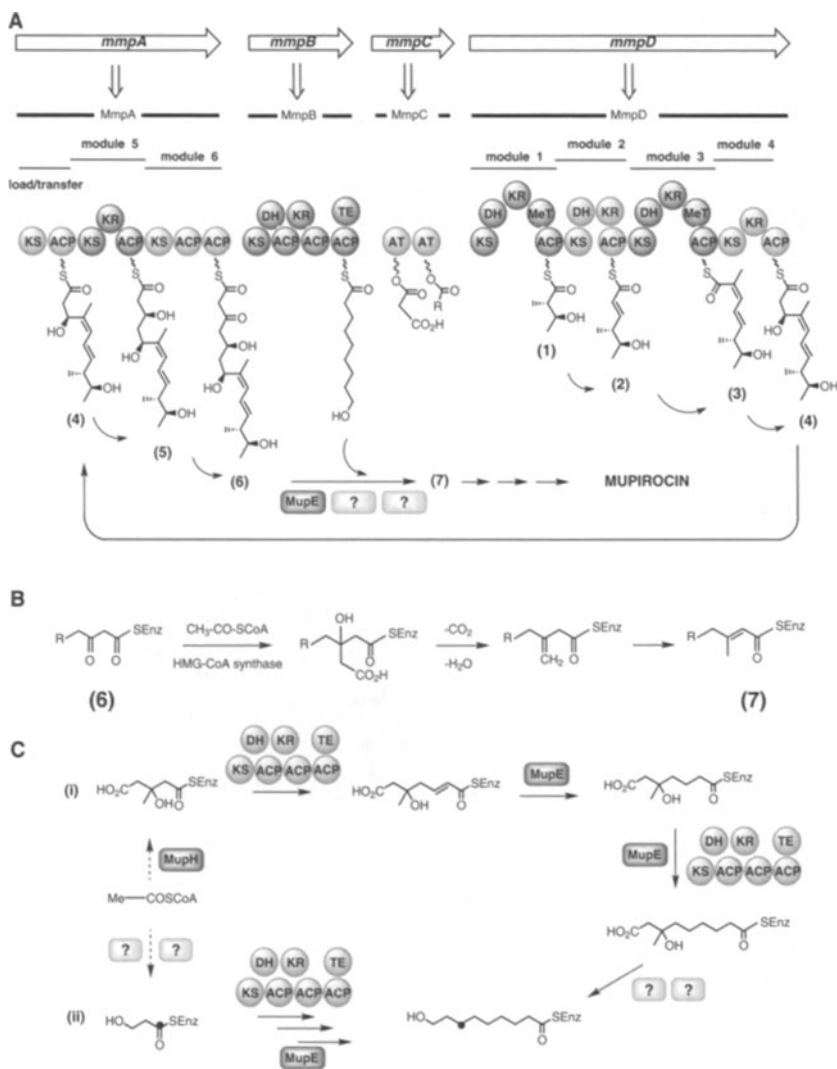


Figure 5. Model for the biosynthesis of mupirocin components. A. Mupirocin biosynthesis model. B. HMG-based model for incorporation of the C15 methyl group in monic acid. C. 9-hydroxynonanoic acid biosynthesis model. (i) 3-hydroxy-3-methylglutaric acid (HMG) starter unit formed from condensation of acetate and acetoacetate catalysed by MupH and extended by two malonate condensations followed by removal of 3-methyl and hydroxyl groups. (ii) Alternatively, a 3-hydroxypropionate starter unit may be extended by three malonate condensations. Acyltransferase (AT), ketosynthase (KS), dehydratase (DH), ketoreductase (KR), methyl transferase (MeT), acyl carrier protein (ACP), thioesterase (TE), enoyl reductase (MupE); HMG-CoA synthase (MupH).

precursor (6) directed by the PKS MmpA, and possibly the HMG-CoA synthase MupH (see below).

MmpD should encode sequential modules that catalyse the first four condensations. The following description refers to carbons by number in the conventional diagrams of mupirocin structure (Figure 5A). Starter and extender units could be activated by MupU and MupQ, which show similarity to acyl-CoA synthases that activate substrates by linking their carboxyl group to the phosphoryl group of AMP. The activated substrate is then transferred to Coenzyme A (CoA). Mupirocin biosynthesis could start with one of the ATs transferring an activated acetyl starter unit from CoA to the 4'-phosphopantetheine arm of ACP8 and then to the thiol group of the active cysteine of KS5 (both in the proposed module 1 of MmpD) in a decarboxylative step. An activated malonyl extender could then be transferred to the vacant ACP8. Alternatively an activated acetyl unit may be transferred direct to KS5. KS5 is proposed to catalyse the first decarboxylative condensation step of acetate and malonate, followed by addition of a methyl group from SAM at C12 by a methyl transferase (MeT) MeT1 (in the proposed module 1 of MmpD) and ketoreduction catalysed by KR3, in the same module, producing a C₅ structure (1). DH2, in the same module, is probably non-functional due to its putative consensus active site (HxxxDxxxxA) differing from other DHs (HxxxGxxxxP). Such non-functional domains have been observed in several modular PKSs, for example, RAPS 1 and 2 in rapamycin biosynthesis in *Streptomyces hygroscopicus*³ and in *pltD* from pyoluteorin biosynthesis cluster in *P. fluorescens* Pf-5⁷⁶. The second cycle of condensation is predicted to be catalysed by MmpD KS6 (module 2) and followed by ketoreduction then dehydration catalysed by KR4 and DH3, respectively (of the same module), producing a C₇ structure (2). The third condensation reaction corresponds to MmpD KS7 (module 3), followed by addition of a methyl group from SAM at C8 by MeT2, reduction and dehydration of the keto group by KR5 and DH4, respectively (again within the same module), producing a C₁₀ structure (3). The fourth condensation step is catalysed by MmpD KS8 (module 4), in the same module, followed by reduction by KR6 producing a C₁₂ structure (4).

Further assembly of MA may be continued by MmpA. A fifth condensation of malonate catalysed by MmpA KS2 (module 5) followed by ketoreduction by KR1, in the same module, should produce a C₁₄ structure (5). The sixth condensation should be catalysed by MmpA KS3 (module 6) and leaving the keto unreduced should produce a C₁₆ β -keto thioester (6). This does not predict a function for KS1 which may not have a completely normal function since it has Gln instead of His in its proposed active site. However, it is necessary for biosynthesis as indicated by the fact that mutation of KS1 gives a Mup⁻ phenotype. Further work will be necessary to establish its role.

The incorporation of a methyl group at C15 could be via condensation of an acetate unit at the β -keto group of structure (6) by the putative HMG-CoA synthase (MupH) (Figure 5B). The resulting carboxylic acid group would undergo decarboxylation followed by dehydration, possibly involving activation of the hydroxyl group for example by phosphorylation producing a terminal alkene CH_2 group. The double bond would rearrange in conjugation with the thioester carbonyl presumably by an isomerase-dependent reaction producing the C_{17} $\alpha\beta$ -unsaturated thioester (7) in which the 15-methyl group is derived from the methyl carbon of the cleaved acetate unit. Further modifications are required to generate MA from structure (7), which would include oxidation and reduction to create the epoxide group and tetrahydropyran ring.

It is much more difficult to predict the source of 9-HN, which is a C_9 fully saturated fatty acid chain. This may be synthesised by a single PKS/FAS with full reduction of the keto groups by ketoreduction, dehydration and enoyl reduction. One possibility is that MupH (HMG-CoA synthase) may catalyse the condensation of acetate and acetoacetate to generate HMG, which would be chain-extended by two condensations of malonate moieties followed by removal of the 3-methyl and hydroxyl groups and reduction of the distal carboxyl group (Figure 5C(i)). However, studies with doubly ^{13}C labelled HMG showed no intact incorporation⁶⁷. Formally 9-HN could be formed from a 3-hydroxypropionate starter unit by three malonate condensations catalysed by a dedicated FAS (Figure 5C(ii)). Preliminary evidence has been obtained for this by synthesis and feeding of $[1-^{13}\text{C}]\text{-3-hydroxypropionate}$ which resulted in specific enrichment (2.5 fold) of $\text{C}7'$ ¹⁰⁵.

If 3-hydroxypropionate is involved, there are several ORFs of unknown function that could be involved. One candidate PKS/FAS for iterative incorporation of the three malonates could be MmpB, which contains KS4, KR2, DH1, ACP5-7 and TE. However, MmpB does not contain an ER domain. MupE shows sequence similarity to ER and therefore could catalyse the final reduction step of 9-HN. Precedence for this possibility is found in lovastatin biosynthesis where the proper functioning of the *lovD* nonaketide synthase (LNKS) requires the accessory *lovC* protein⁵. It is also possible that the organism makes use of the ER used in endogenous fatty acid metabolism, as has been suggested for prodigiosin biosynthesis in *Streptomyces coelicolor*¹⁸.

The TE domain at the end of MmpB would fit with this model for 9-HN biosynthesis being involved with release of either 9-HN or mupirocin or with esterification of 9-HN and MA. Alternatively, the C_9 moiety may be derived from a pathway outside of the cluster. It could also involve other enzymes from within the cluster, for example, *mupB* and *mupG* (3-oxoacyl-ACP synthases), and *mupD* and *mupS* (3-oxoacyl-ACP reductases) for which roles have not been assigned.

Pseudomonic acid B contains a hydroxyl group at C8, which may be a result of further oxidative modification. Pseudomonic acid C has an alkene at C10–C11 instead of an epoxide, which may be due to lack of processing by

MupO (putative P450 oxygenase). Pseudomonic acid D has an unsaturated C₉ chain (alkene at C4'–C5'), which may arise from the second malonate condensed with 3-hydroxy-propionate not being fully reduced by ER.

3.4. Regulation of Mupirocin Biosynthesis

Mupirocin biosynthesis is under the control of a quorum-sensing regulatory system. MupR shows similarity to LasR/LuxR (transcriptional activators), and MupI to LasI/LuxI (synthases for *N*-acyl homoserine lactones [AHL] that activate LasR/LuxR)^{31, 38, 41}. Mutational inactivation of MupR and MupI and *xyIE* reporter assays revealed that MupI generates a diffusible substance that stimulates transcription of the *mup* cluster in the presence of MupR during late exponential and stationary phase. Furthermore, the MupI product can activate the LasR/LasI system that is preferentially activated by longer-chain AHLs (C₁₀–C₁₄), and a MupI[−] phenotype could be complemented by exogenous feeding with culture extract of *Pseudomonas aeruginosa* that should contain cognate AHL for LasR induction (3-oxo-C12-HSL)³¹.

A putative MupR box upstream of *mupA* was identified that may activate transcription from the *mupA* promoter³¹. No potential internal promoters were identified either by similarity to consensus sequences for any of the known sigma factors or the putative MupR binding site.

4. PEDERIN

4.1. Pederin Activity and Structure

Pederin is produced by unculturable symbionts of *Paederus* and *Paederidus* beetles^{54, 55}. 16sRNA typing indicates that the symbiotic bacterium of *Paederus fuscipes* is a close relative of *P. aeruginosa*⁵³. Furthermore, sequence analysis of a >110 kb region encompassing the pederin biosynthesis cluster revealed that most ORFs outside the pederin cluster, including almost all housekeeping genes, exhibit >80% identity at the nucleotide level with genes from *P. aeruginosa*⁸⁶. Although members of the pederin metabolite family have previously been isolated from marine sponges, pederin is exclusively isolated from terrestrial *Paederus* and *Paederidus* beetles. The beetles utilise pederin as a chemical defence weapon against predators⁵⁶. More importantly, however, the pederin group of polyketide metabolites are highly active anti-tumour compounds, in particular pederin, and mycalamide A and B^{17, 90}.

Pederin (C₂₅H₄₅O₉N) is a polyketide metabolite synthesised from malonyl- and methylmalonyl-CoA units, with glycine incorporated by NRPS. It consists of two modified pyran rings one of which is dimethylated⁶⁸ (Figure 6).

4.2. Pederin Biosynthetic Cluster

Degenerate PCR based on motifs of KS domains was used to amplify sequences that were then used to screen a total DNA library from *P. fuscipes*⁸⁶. A 53.8 kb region was subsequently sequenced (Accession no. AY059471), which revealed ORFs showing homology with Type I PKS genes, designated as *ped* genes. The pederin biosynthesis cluster contains eight ORFs *pedA-pedH* that encode both Type I and Type II PKS (Figure 6). PedF and PedH show similarity to a mixed Type I modular PKSs/NRPSs. These, like the mupirocin MMPs, lack AT domains integrated within the PKS modules. Instead, the cluster contains two monodomain ATs encoded by *pedC* and *pedD* that presumably act *in trans*. Interestingly, PKSs that lack ATs are more closely related to each other than other Type I PKSs⁸⁶. PedA and PedE show similarity to methyl transferases, PedB to FMN dependent oxidoreductases, and PedG with FAD-dependent monooxygenases. Apart from lacking the ATs, PedF appears to be perfectly colinear with pederin structure. However, the cluster is missing a small PKS that encodes the first three modules, a protein for the attachment of exomethylene group and an enzyme for catalysing the formation of the aminal moiety⁸⁶.

The PKS/NRPS encoded by PedH would result in the biosynthesis of a much larger polyketide/peptide metabolite than required for pederin. This may indicate that the entire cluster when originally acquired encoded a larger sponge-type metabolite with which this hypothetical intermediate has similarity⁸⁶. During the course of evolution of the pederin biosynthesis pathway, the oxygenase gene *pedG* may have inserted into the cluster destroying a module and resulting in premature termination of the PKS elongation at module PedF6 with subsequent release of a pederin precursor by oxidative cleavage. The pederin cluster is flanked by transposase pseudogenes, which suggests that the pathway may have been acquired through horizontal transfer and insertion into the genome by a transposition event.

4.3. Pederin Biosynthesis Model

Piel⁸⁶ has proposed a model for pederin biosynthesis based on the structure of pederin and the domain order of PedF and PedH and the positioning of PedG (Figure 6). The first as yet unidentified modules should catalyse the condensation of a C₅ intermediate, which would be loaded onto the ACP of the first module of PedF (PedF1) and extended by a C₂ unit. This C₆ intermediate would be transferred to PedF2, which is a NRPS that would catalyse the attachment of glycine as directed by the non-ribosomal code of the adenylation domain. PedF3 catalyses extension by C₂ and reduction of the β-keto to a hydroxyl group. PedF4 catalyses extension by another C₂ unit, which is again

reduced at the β -keto to a hydroxyl group. The methyl transferase domain within this module would catalyse the attachment of a germinal dimethyl group. PedF5 would extend by another C_2 and then reduce to an epoxide. The interesting duplicated DH domain would catalyse formation of the pyran ring. PedF6 would extend by another C_2 with reduction of the β -keto to a hydroxyl group. There are then two alternative routes that pederin biosynthesis may follow. Through route A the intermediate, bound as a thioester to PedF6, would be cleaved by the oxidative activity of PedG. Alternatively via route B, the intermediate may be further elongated and processed by the PKS/NRPS enzymatic domains of PedH, which would result in the generation of a much longer polyketide/peptide metabolite than pederin, as described above. This intermediate could then be cleaved by PedG, in a Bayer-Villiger oxidation, to generate the pederin precursor and another fragment. Pederin contains four O-methyl groups of which at least two could be attached by the methyl transferase activity of PedA and PedE.

5. CORONATINE

5.1. Coronatine Activity and Structure

Coronatine is a phytotoxin produced by several strains of *Pseudomonas syringae* pathovars (pvs.) including pvs. *atropurpurea*, *glycinea*, *maculicola*, *morsprunorum* and *tomato* that infect ryegrass, soybean, crucifers, *Prunus* spp. and tomato, respectively¹². Disease symptoms include diffuse chlorosis on a wide range of plants, whereby yellow spots arise from lack of chlorophyll formation, and in some plant species hypertrophy, whereby tissues become enlarged, inhibition of root elongation and stimulation of ethylene production⁵⁷. This phytotoxin is also dealt with elsewhere in this (Bender and Scholz-Schroeder, Chapter 4, Volume 2) but is included here for completeness.

Coronatine ($C_{18}H_{25}O_4N$) consists of a polyketide moiety, coronafacic acid (CFA), linked via an amide bond to coronamic acid (CMA), an ethylcyclopropyl amino acid derived from isoleucine (Figure 7)⁴⁹. ^{13}C -labelled substrate feeding experiments revealed that CFA is derived from one pyruvate, one butyrate and three acetate units⁸³. CFA and CMA are discrete intermediates secreted by COR producers, and both are required for coronatine phytotoxic activity^{74, 114, 119}. Although coronatine is the major coronafacoyl compound produced by *P. syringae*, others may be produced by coupling CFA with different amino acid moieties, generating, for example, coronafacoylisoleucine, coronafacoylalloisoleucine, coronafacoylvaline (CFV) and norcoronatine⁷¹.

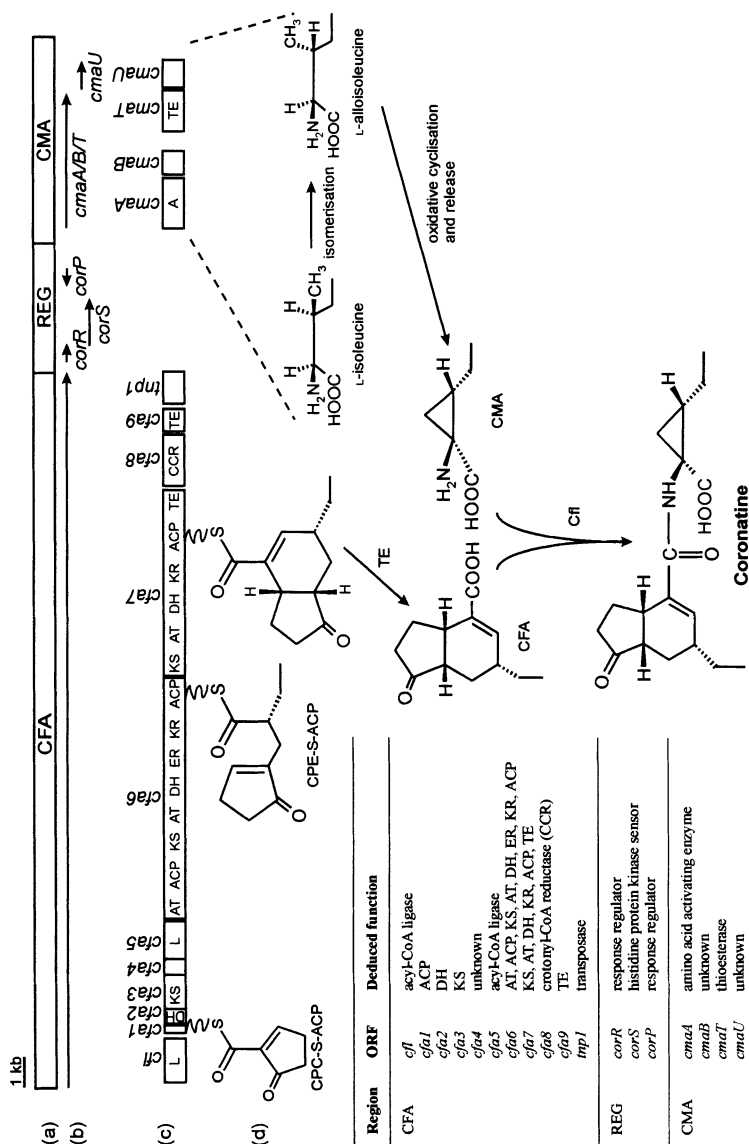


Figure 7. Organisation of the coronatine biosynthesis cluster and model of biosynthesis. (a) Region organisation; (b) transcripts; (c) ORFs; (d) biosynthesis model. Coronafacic acid (CFA), regulatory region (REG), coronamic acid (CMA), acyl-CoA ligase (L), 2-carboxy-2-cyclopentenone (CPC), acyl carrier protein (ACP), dehydratase (DH), ketosynthase (KS), acyltransferase (AT), enoyl reductase (ER), ketoreductase (KR), 2-[1-oxo-2 cyclopenten-2-ylmethyl] butanoic acid (CPE), thioesterase (TE), crotonyl-CoA reductase (CCR), amino acid activating enzyme (A).

Coronatine is believed to act as a mimic of the octadecanoid-derived signalling molecules in higher plants. At low levels, jasmonic acid is a signal transducer in higher plant's defence against herbivore or pathogen^{32, 42}. However, at higher levels, it induces symptoms resembling coronatine activity: chlorophyll degradation, accelerated senescence and induced production of ethylene⁹⁸. Molecular overlays reveal that coronatine is a close structural analogue of the jasmonic acid precursor 12-oxo-phytodienoic acid (OPDA)¹¹⁴. Furthermore, coronatine and OPDA both induce the same positive response in a tendril curvature assay, where jasmonates substitute for a mechanical stimulus and induce coiling¹¹⁴.

5.2. Coronatine Biosynthetic Cluster

The COR biosynthetic cluster may be either chromosomally or plasmid encoded. *P. syringae* pvs. *atropurpurea*, *glycinea*, *morsprunorum* and *tomato* generally contain the COR cluster on a large plasmid, whereas it is usually chromosomally encoded in pv. *maculicola*^{13, 24, 61}. The COR biosynthesis cluster has been most extensively studied in *P. syringae* pv. *glycinea* PG4180, encoded on a 90 kb plasmid p4180A^{11, 119}. Non-coronafacoyl producing strains *P. syringae* pv. *syringae* PS51 and PS61 transformed with p4180A acquire the ability to produce coronatine¹¹. The COR biosynthetic cluster was divided into functional regions through Tn5 mutagenesis, which isolated COR⁻, CFA⁻ and CMA⁻ mutants whose defects could be rectified by exogenous feeding with CFA and CMA, and complemented by expression *in trans* of the coupling region and regions required for CMA and CFA biosynthesis^{11, 88, 108, 109, 119}.

The 32 kb COR biosynthetic cluster consists of two distinct regions responsible for CMA and CFA biosynthesis, which are separated by a 3.4 kb regulatory region (Figure 7). The 6.95 kb CMA region contains four separate ORFs: *cmaA*, *cmaB*, *cmaT* and *cmaU* that are thought to synthesise CMA via a NRPS thiotemplate mechanism^{16, 108} (Accession no. U14657). *cmaA*, *cmaB* and *cmaT* are co-transcribed as a 5 kb transcript, whereas *cmaU* is transcribed separately¹⁰⁸. CmaA shows sequence similarity to enzymes that activate amino acids by adenylation and bind them as thioesters. CmaT is related to known TEs, but putative functions have not yet been assigned for CmaB and CmaU. The CFA region contains ten ORFs: *cfl*, and *cfa1-cfa9*, which are co-transcribed from a single promoter upstream of *cfl* as a single 19 kb transcript, and show homology with enzymes involved in polyketide synthesis (Figure 7)^{63, 85, 87, 88} (Accession nos. U56980, AF098795, AF061506). Another ORF, *tnp1*, is present in the CFA cluster which has no function in COR biosynthesis but shows homology with transposase encoded by IS5⁸⁸. Multiple insertion sequences flanking the COR biosynthesis cluster have also been identified in p4180A, and may have been involved in its acquisition and assembly². The CMA and CFA sections may have

evolved and been acquired independently, bringing them together under the co-ordinated control of the regulatory region. This region contains three ORFs *corR*, *corP* and *corS*^{2, 88} (Accession nos. U33326, U33327).

5.3. Coronatine Biosynthesis Model

A model for CFA biosynthesis has been proposed which involves both mono- and multifunctional enzymes (Figure 7)⁸⁷. Cfl, which shows homology with acyl-CoA ligases that activate specific carboxylic acid substrates by adenylation, has been shown to be required for CFA biosynthesis⁸⁹. Incorporation of ¹³C-labelled pyruvic acid showed that CFA is synthesised from the C2 and C3 of pyruvate⁸³. It has therefore been proposed that pyruvate may be converted to α -ketoglutarate via oxaloacetate before incorporation, and that Cfl and Cfa1-5 could convert α -ketoglutarate, as the starter unit, to an as yet unidentified compound, which could then be loaded onto the PKS Cfa6. α -ketoglutarate may be decarboxylated to produce succinic semialdehyde, which would contain the C2 and C3 of pyruvate but not the C1^{82, 87}. Succinic semialdehyde (C₄) bound as a thioester to Cfa1 (ACP) may then be extended by acetate (C₂) from a malonyl-CoA extender unit catalysed by Cfa3 (KS), cyclised by an as yet unidentified protein, possibly Cfa4, then dehydrated by Cfa2 (DH) to generate 2-carboxy-2-cyclopentenone (CPC). CPC may then be transferred directly to the PKS Cfa6. Alternatively it may be released from Cfa1 (ACP) by Cfa9 (TE) and then activated as a CoA ester by the acyl-CoA ligases Cfl or Cfa5 and loaded onto the N-terminal AT and ACP domains of Cfa6, which resemble the loading domain of the DEBS module 1 from Type I PKS system for erythromycin biosynthesis by *Saccharopolyspora erythraea*⁸⁷. Cfa8 shows homology with crotonyl-CoA reductase, which catalyses conversion of acetoacetyl-CoA to butyryl-CoA, that is used as a C₄ extender unit in polyketide synthesis^{39, 87}. Therefore, CFA biosynthesis may also require a butyryl-CoA precursor. Cfa6 is a multifunctional PKS enzyme that contains discrete domains that could catalyse extension of the bound intermediate by butyrate (C₄) from an ethylmalonyl-CoA extender unit, followed by complete reduction of the β -keto group. The Cfa6-bound intermediate if released by TE activity would generate 2-[1-oxo-2-cyclopenten-2-ylmethyl] butanoic acid (CPE), which has been isolated at low levels from fermentation broth⁷³. Alternatively transfer to Cfa7, another multifunctional PKS enzyme, could then be followed by extension by another acetate (C₂) unit from malonyl-CoA, reduction and dehydration of the β -keto group. CFA could then be released by the TE activity of either Cfa7 or Cfa9. Cfa9 has been shown to be non-essential for COR biosynthesis, but may increase release⁸⁸.

A model has also been proposed for CMA biosynthesis which utilises a NRPS mechanism^{83, 108}. The substrate amino acid is as yet unknown, but may

be L-isoleucine or L-alloisoleucine since CMA biosynthesis requires isomerisation of L-isoleucine to L-alloisoleucine. This could be adenylated by CmaA and bound as a thioester via a phosphopantetheine arm. An intermediate of CMA or the final product may then be released from CmaA by the TE activity of CmaT. Parry *et al.*⁸³ predicted that an oxidative cyclisation reaction was required to form the cyclopropane ring, which may utilise an active ferrous site. The N-terminus of CmaA has a spatial arrangement of amino acid residues that resemble the ferrous active sites of various nonheme iron(II) proteins¹⁰⁸. Thus, CmaA may be involved in the oxidative cyclisation of L-alloisoleucine. Incorporation of deuterium labelled CMA shows that it is a discrete intermediate and that cyclisation must occur prior to formation of the amide bond between CFA and CMA⁸³.

The acyl-CoA ligase Cfl is presumed to be also involved in coupling CFA and CMA. Expression *in trans* of Cfl in a non-COR producing strain that was fed with exogenous CFA and CMA conferred ligation activity and led to the production of coronatine⁸⁹. Liyanage *et al.*⁶³ proposed that Cfl may activate CFA by adenylation prior to coupling with CMA. However, to date, attempts to isolate CFA adenylated by purified Cfl have proven unsuccessful. The specificity of Cfl-encoded coupling varies between different coronafacoyl-producing strains. For example, *P. syringae* pv. *glycinea* PG4180 prefers coupling CFA with CMA, and couples CFA with valine, isoleucine and alloisoleucine at reduced levels¹¹⁹. However, *P. syringae* pv. *atropurpurea* 4328 produces equal amounts of COR and CFV suggesting equal affinity for CMA and valine⁷².

5.4. Regulation of Coronatine Biosynthesis

The regulatory region contains three ORFs *corR*, *corS* and *corP*. The deduced amino acid sequence of CorS shows homology with histidine protein kinases that act as environmental sensors, whereas CorR and P show homology with response regulators that are members of two-component regulatory systems¹¹⁰. Response regulators typically contain an N-terminal receiver domain that is the site of phosphorylation, and a C-terminal effector domain with a helix-turn-helix (HTH) DNA binding motif^{80, 81}. Both of these domains are present in CorR, but only the N-terminal receiver domain is present in CorP. Sequence comparisons of the N-terminal domains of CorR and CorP show that they are almost identical, which suggests that they share specificity for the same phosphodonor¹¹⁰. CorR has been shown to be a positive activator of COR gene expression that binds to the *cfl* and *cmaA* promoter regions^{62, 84, 110, 113}. COR biosynthesis has also been shown to be thermoregulated. COR production and *cfl*, *cmaA*, *cmaU* and *corS* gene expression are maximal at 18°C with little or no production or expression at 28°C^{62, 89, 91, 108, 110}. The pathway-specific

CorRPS system, thermoregulation, global regulation by σ^{54} , plant stimulation of COR production by DC3000 and differences between DC3000 and PG4180 in the role of COR in plant microbe interactions are discussed in more detail in another chapter in this book (Bender and Scholz-Schroeder, Chapter 4, Volume 2).

6. 2,4-DIACETYLPHLOROGLUCINOL (DAPG)

6.1. DAPG Activity and Structure

2,4-Diacetylphloroglucinol (DAPG), produced by various fluorescent *Pseudomonads*^{51, 60, 77, 100, 112}, has antifungal, antibacterial, antihelminthic and phytotoxic properties^{15, 51, 112}. This secondary metabolite is also dealt with elsewhere in this book (Morrissey *et al.*, Chapter 21) but is included here for completeness. DAPG is recognised as the key secondary metabolite associated with biological control of a range of soilborne fungal plant pathogens that cause root and seedling diseases on a variety of crops. For example, DAPG is important in *P. fluorescens* CHA0 suppression of black root rot of tobacco caused by *Thielaviopsis basicola*^{51, 103}, take-all disease of wheat caused by *Gaeumannomyces graminis* var. *tritici*⁵¹, and Fusarium crown and root rot of tomato²⁹. Interestingly DAPG or its derivatives have also been indicated as potential candidates for anti-methicillin resistant *S. aureus* compounds⁵⁰. The mode of action of DAPG has not yet been reported.

DAPG is a phenolic compound of polyketide origin (Figure 8). Monophloroglucinol (MAPG) and DAPG have been identified from culture extracts of Q2-87 and F113^{7, 99}. MAPG is believed to be the precursor of DAPG, which has an additional acetyl group. Evidence for this comes from the description of transacetylation of MAPG to DAPG by cell extracts of F113⁹⁹.

6.2. DAPG Biosynthetic Cluster

A 6.5 kb fragment from *P. fluorescens* Q2-87 conferred the ability to produce DAPG on non-DAPG producing *Pseudomonas* strains⁶. Tn3 mutagenesis revealed a 5 kb locus required for DAPG biosynthesis, which is highly conserved among DAPG producing *Pseudomonads*^{6, 52}.

Sequence analysis revealed six ORFs *phlA*, *phlB*, *phlC*, *phlD*, *phlE* and *phlF* as three transcriptional units (Accession no. U41818)⁷. *phlACBD* are expressed as an operon from a promoter upstream of *phlA* and are flanked on either side by *phlE* and *phlF* (Figure 8). PhlA shows similarity to FabH, a β -ketoacyl-ACP synthase III (KASIII) from *E. coli*, which catalyses the

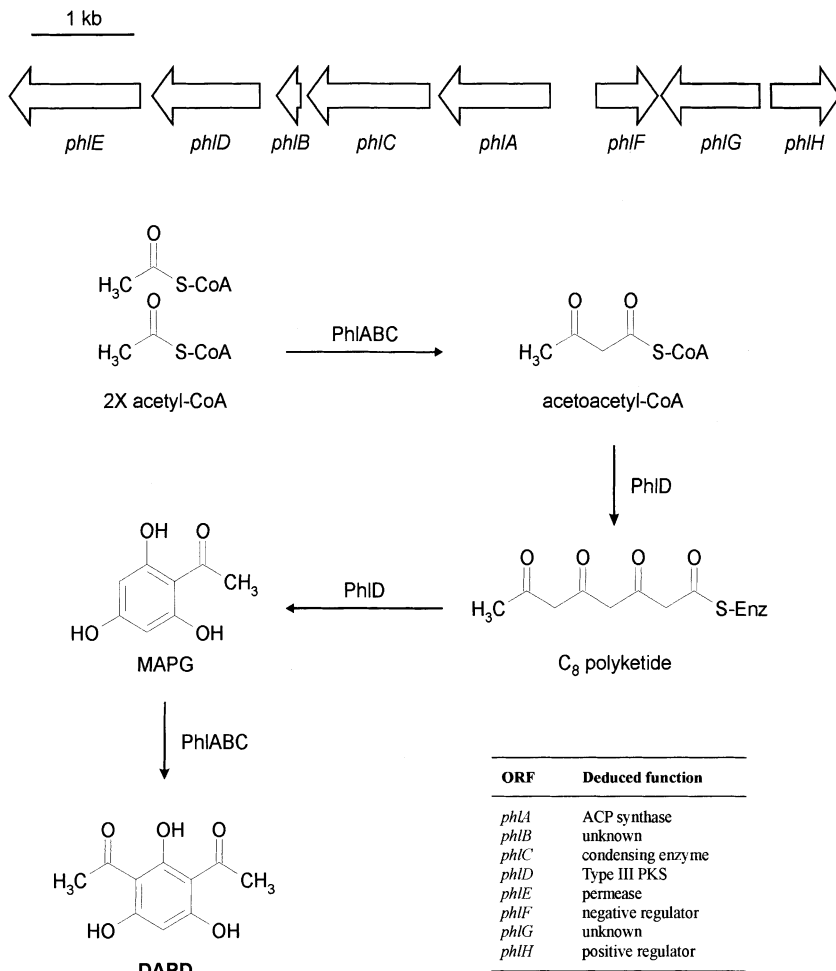


Figure 8. Organisation of the 2,4-diacetylphloroglucinol biosynthesis cluster and model of biosynthesis. 2,4-diacetylphloroglucinol (DAPG), monophloroglucinol (MAPG).

condensation of acetyl-CoA with malonyl-ACP to form acetoacetyl-ACP in Type II fatty acid synthesis¹⁰⁷. However, PhlA lacks the active site cysteine characteristic of condensing enzymes. FabH also has transacylase activities, whereby an acetate moiety is transferred from acetyl-CoA to acetyl-ACP¹⁰⁷. PhlC contains a putative carboxylic acid-CoA binding site typical of condensing enzymes. PhlB has not been assigned a putative function. PhlD is a PKS that shows similarity to plant chalcone and stilbene synthases which are Type III PKSs. Chalcone synthases (CHS) consist of homodimeric proteins that perform condensation and cyclisation steps required for biosynthesis of phenolic

compounds. PhlE shows similarity with a multidrug efflux transporter from the membrane permease superfamily and is associated with a red pigment present in media of DAPG producing strains⁶. PhlF contains a HTH domain and is a pathway-specific repressor of DAPG biosynthesis²⁶.

Sequence analysis of CHAO revealed two additional ORFs *phlG* and *phlH* adjacent to *phlF*, which are not related to the previous *phl* genes (Accession no. AF207529)⁹⁵. PhlH shows similarity to regulatory proteins, but no putative function has been assigned to PhlG. Tn5 mutagenesis of *phlH* knocked out MAPG and DAPG production⁹⁵.

6.3. DAPG Biosynthesis Model

Knockouts, exogenous feeding and expression in *E. coli* revealed that PhlD is responsible for the biosynthesis of the DAPG precursor MAPG, and that PhlACB are necessary for the conversion of MAPG to DAPG and may also be required for the biosynthesis of MAPG⁷. PKS biosynthesis of DAPG involves the condensation of three acetyl-CoA units with one malonyl-CoA to produce MAPG which is then transacetylated to DAPG⁹⁹.

Bangera and Thomashow⁷ postulate that PhlC binds acetyl-CoA and then PhlACB catalyse a condensation reaction with another acetyl-CoA unit to provide a linear primer unit, acetoacetyl-CoA (Figure 8). The Type III PKS PhlD then catalyses the condensation of this primer molecule with extender units and cyclisation to generate the C₈ intermediate MAPG, which is then converted to DAPG by the activity of PhlACB. This acetylation reaction corresponds to MAPG acetyl-transferase activity, which has been described in cell extracts of F113⁹⁹.

6.4. Regulation of DAPG Biosynthesis

Translational and transcriptional fusions of a *lacZ* reporter with *phlA* showed that in *P. fluorescens* CHAO, *phlA* expression was maximal during late exponential phase growth, that *phlA* expression was autoinduced by DAPG, repressed at the transcriptional level by PhlF and also repressed by other bacterial metabolites pyoluteorin and salicylate and the fungal pathogenicity factor fusaric acid⁹⁵. PhlF is required for autoinduction by DAPG and repression by these three metabolites. If exogenous phloroglucinol is added to F113 cultures in early exponential phase, it stops growth and replication. Therefore, it may be self-inhibitory to accumulate DAPG early in the growth phase, and hence it has been postulated that PhlF may play a role in the prevention of accumulation of DAPG during this phase²⁶. PhlF is indeed expressed at high levels during early growth phase¹. Furthermore, PhlF binds to an inverted repeat sequence *phlO* downstream of the *phlA* transcriptional start site¹.

Regulators of DAPG biosynthesis act directly with PhlF by modulating its binding to the *phO* operator. Autoinduction by DAPG may result from DAPG destabilising the PhlF-*phO* complex thus allowing expression of the *phlACBD* operon, while repressors may stabilise it and inhibit expression¹. The pathway is also positively modulated by PhlH⁹⁵.

In addition to these pathway-specific regulators, DAPG biosynthesis is also influenced by environmental factors such as carbon source^{28, 100, 120}, and is under the control of the two component GacS/GacA system, which are positive regulators of expression of *phlACBD* and *phlF*⁴³, and use of alternative sigma factors^{69, 92, 94}. These regulatory systems are discussed in further detail in Chapter 21 in this book (Morrissey *et al.*).

7. GENERAL DISCUSSION

The collection of systems described in this chapter are connected by the fact that at least part of the chemical products of the pathways they encode are synthesised by a mechanism involving one or more PKS enzymes. The size of the genetic regions involved varies from the 6.5 kb region of DAPG to the 75 kb region needed for mupirocin biosynthesis. Within these regions the diversity of the pathways is quite remarkable. In the Introduction we explained the key differences between the Type I, II and III PKS systems and the relationship to the NRPS mechanism. Across the collection of systems we find all these represented in different combinations. Type I PKS are found in CFA, pyoluteorin, mupirocin, pederin; Type II in CFA, DAPG, mupirocin and pederin; Type III in DAPG while NRPS is found in CMA, pyoluteorin and pederin. This illustrates the way that pathways can be built up from diverse modules so long as the carrier proteins (ACPs or PCPs) can be loaded, the extender unit they carry can act as the recipient for the growing chain and that the carrier protein can then pass the growing chain on to the next synthase or release its product, possibly with the help of a TE. This flexibility in the assembly of natural pathways underpins the current experiments to create new pathways by combinatorial genetics using the components found in nature. The collection of systems described also illustrates the diversity of tailoring enzymes that can act as part of the biosynthetic pathways and provides yet more potential for building new products.

Although most of the effort to date in such manipulations has been with the actinomycetes, the diversity of pathway types and pathways components found across *Pseudomonas* species means that there is considerable scope for similar approaches in this genus. Genetic manipulation within many *Pseudomonas* species is quite straightforward and it may well be that the fact that the organisms are non-filamentous provides advantages for growing the bacteria and overproducing their products. The fact that the genome sequences

of a number of *Pseudomonas* species are now available, or will be in the near future, will facilitate identification of the accessory gene systems that are needed for optimum activity of the pathways and underpin the rational approach to pathway manipulation. The sequences are also revealing additional gene systems that can be used as a part of the repertoire of genes for the combinatorial approaches.

In addition to the creation of GMOs with new activities, the fact that horizontal gene transfer has obviously played a significant role in the evolution of the various pathways described and that at least some of the pathways are encoded on mobile gene systems — plasmids, transposable elements or phenotypic islands — means that there is scope for creation of new strains by *in vivo* genetic manipulation. Thus, rational mixing of naturally occurring strains with the potential to cause spread and recombination of the various component gene systems, could create strains that should be acceptable for release to natural environments. The approaches described in *Rhizobium* for detecting and enriching for rare recombinants simply by the ability to detect them by PCR designed for the desired combination of DNA sequences suggests an approach that could be used. Thus, development of new biocontrol strains could benefit from PKS combinatorial genetics as well as the production of novel products for pharmaceutical use where production would take place under contained conditions. Therefore, the search for new pathways including PKS systems in *Pseudomonas* species has great potential for biotechnology applied to medicine as well as agriculture. The lessons learnt from the systems described in this chapter should provide the basis for predicting functions of uncharacterised systems, and hence facilitate the rapid exploitation of this new information.

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REFERENCES

1. Abbas, A., Morrissey, J.P., Marquez, P.C., Sheehan, M.M., Delany, I.R., and O'Gara, F., 2002, Characterisation of interactions between the transcriptional repressor PhlF and its binding site at the *phlA* promoter in *Pseudomonas fluorescens* F113. *J. Bacteriol.*, 184:3008–3016.
2. Alarcon-Chaidez, F.J., Penaloza-Vazquez, A., Ullrich, M., and Bender, C.L., 1999, Characterization of plasmids encoding the phytotoxin coronatine in *Pseudomonas syringae*. *Plasmid*, 42:210–220.
3. Aparicio, J.F., Molanar, I., Schwecke, T., Koning, A., Haydock, S.F., Khaw, L.E., Staunton, J., and Leadlay, P.F., 1996, Organisation of the biosynthetic gene cluster for rapamycin in

- Streptomyces hygroscopicus*: Analysis of the enzymatic domains in the modular polyketide synthase. *Gene*, 169:9–16.
4. Armstrong, S.M. and Patel, T.R., 1993, Abiotic conversion of dihydrophloroglucinol to resorcinol. *Can. J. Microbiol.*, 39:899–902.
 5. Auclair, K., Kennedy, J., Hutchinson, C.R., and Vederas, J.C., 2001, Conversion of cyclic nonaketides to lovastatin and compactin by a *lovC* deficient mutant of *Aspergillus terreus*. *Bioorg. Med. Chem. Lett.*, 11:1527–1531.
 6. Bangera, M.G. and Thomashow, L.S., 1996, Characterisation of a genomic locus required for synthesis of the antibiotic 2,4-diacetylphloroglucinol by the biocontrol agent *Pseudomonas fluorescens* Q2-87. *Mol. Plant Microbe Interact.*, 9:83–90.
 7. Bangera, M.G. and Thomashow, L.S., 1999, Identification and characterization of a gene cluster for synthesis of the polyketide antibiotic 2,4-diacetylphloroglucinol from *Pseudomonas fluorescens*, Q2-87. *J. Bacteriol.*, 181:3155–3163.
 8. Bate, N., Butler, A.R., Smith, I.P., and Cundliffe, E., 2000, The mycarose-biosynthetic genes of *Streptomyces fradiae* producer of tylosin. *Microbiology*, 146:139–146.
 9. Bate, N. and Cundliffe, E., 1999, The mycinose-biosynthetic genes of *Streptomyces fradiae*, producer of tylosin. *J. Ind. Microbiol. Biotechnol.*, 23:118–122.
 10. Beck, J., Ripka, S., Siegner, A., Schiltz, E., and Schweizer, E., 1990, The multifunctional 6-methylsalicylic acid synthase gene of *Penicillium patulum*—its gene structure relative to that of other polyketide synthases. *Eur. J. Biochem.*, 192:487–498.
 11. Bender, C.L., Liyanage, H., Palmer, D., Ullrich, M., Young, S., and Mitchell, R., 1993, Characterization of the genes controlling the biosynthesis of the polyketide phytotoxin coronatine including conjugation between coronafacic and coronamic acid. *Gene*, 133:31–38.
 12. Bender, C.L., Palmer, D., Penaloza-Vazquez, A., Rangaswamy, V., and Ullrich, M., 1996, Biosynthesis of coronatine, a thermoregulated phytotoxin produced by the phytopathogen *Pseudomonas syringae*. *Arch. Microbiol.*, 166:71–75.
 13. Bender, C.L., Young, S.A., and Mitchell, R.E., 1991, Conservation of Plasmid DNA sequences in coronatine-producing pathovars of *Pseudomonas syringae*. *Appl. Environ. Microbiol.*, 57:993–999.
 14. Binnie, D., Warren, M., and Butler, M.J., 1989, Cloning and heterologous expression in *Streptomyces lividans* of *Streptomyces rimosus* genes involved in oxytetracycline biosynthesis. *J. Bacteriol.*, 171:887–895.
 15. Bowden, K., Broadbent, J.L., and Ross, W.J., 1965, Some simple antihelmintics. *Br. J. Pharmacol.*, 24:717–724.
 16. Budde, I.P., Rohde, B.H., Bender, C.L., and Ullrich, M.S., 1998, Growth phase and temperature influence promoter activity, transcript abundance, and protein stability during biosynthesis of the *Pseudomonas syringae* phytotoxin coronatine. *J. Bacteriol.*, 180:1360–1367.
 17. Burres, N.S. and Clement, J.J., 1989, Antitumor-activity and mechanism of action of the novel marine natural products mycalamide A and mycalamide B and onnamide. *Cancer Res.*, 49:2935–2940.
 18. Cerdeno, A.M., Bibb, M.J., and Challis, G.C., 2001, Analysis of the prodiginin biosynthesis gene cluster of *Streptomyces coelicolor* A3(2): New mechanism for chain initiation and termination in modular multienzymes. *Chem. Biol.*, 8:817–829.
 19. Chain, E.B. and Mellows, G., 1977, Pseudomonic acid. Part 1. The structure of pseudomonic acid A, a novel antibiotic produced by *Pseudomonas fluorescens*. *J. Chem. Soc. Perkin Trans.*, 1:294–309.
 20. Chain, E.B. and Mellows, G., 1974, Structure of pseudomonic acid, an antibiotic from *Pseudomonas fluorescens*. *J. Chem. Soc.-Chem. Commun.*, 847–848.
 21. Clayton, J.P., Luk, K., and Rogers, N.H., 1979, The chemistry of pseudomonic acid. Part 2. The conversion of pseudomonic acid A into monic acid A and its esters. *J. Chem. Soc. Perkin*, 1:308–313.

22. Clayton, J.P., O'Hanlon, P.J., and Rogers, N.H., 1980, The structure and configuration of pseudomonic acid C. *Tetrahedron Lett.*, 21:881–884.
23. Cookson, B.D., 1998, The emergence of mupirocin resistance: A challenge to infection control and antibiotic prescribing practice. *J. Antimicrob. Chemother.*, 41:11–18.
24. Cuppels, D.A. and Ainsworth, T., 1995, Molecular and physiological characterisation of *Pseudomonas syringae* pv. *tomato* and *Pseudomonas syringae* pv. *maculicola* strains that produce the phytotoxin coronatine. *Appl. Environ. Microbiol.*, 61:3530–3536.
25. Cuppels, D.A., Howell, C.R., Stipanovic, R.D., Stoessl, A., and Stothers, J.B., 1986, Biosynthesis of pyoluteorin—a mixed polyketide-tricarboxylic acid cycle origin demonstrated by [1,2-C-13(2)]acetate incorporation. *Zeitschrift für Naturforschung C.J. Biosci.*, 41:532–536.
26. Delany, I., Sheehan, M.M., Fenton, A., Bardin, S., Aarons, S., and O'Gara, F., 2000, Regulation of production of the antifungal metabolite 2,4-diacetylphloroglucinol in *Pseudomonas fluorescens* F113: Genetic analysis of *phlF* as a transcriptional repressor. *Microbiology*, 146:537–546.
27. Donadio, S., Staver, M.J., McAlpine, J.B., Swanson, S.J., and Katz, L., 1991, Modular organisation of genes required for complex polyketide biosynthesis. *Science*, 252:675–679.
28. Duffy, B.K. and Defago, G., 1999, Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. *Appl. Environ. Microbiol.*, 65:2429–2438.
29. Duffy, B.K. and Defago, G., 1997, Zinc improves biocontrol of *Fusarium crown* and root rot of tomato by *Pseudomonas fluorescens* and represses the production of pathogen metabolites inhibitory to bacterial antibiotic biosynthesis. *Phytopathology*, 87:1250–1257.
30. El-Sayed, A.K., Hotherhall, J., Cooper, S.M., Stephens, E., Simpson, T.J., and Thomas, C.M., 2003, Characterisation of the mupirocin biosynthesis gene cluster from *Pseudomonas fluorescens* NCIMB 10586. *Chem. Biol.*, 10:419–430.
31. El-Sayed, A.K., Hotherhall, J., and Thomas, C.M., 2001, Quorum-sensing-dependent regulation of biosynthesis of the polyketide antibiotic mupirocin in *Pseudomonas fluorescens* NCIMB 10586. *Microbiology*, 147:2127–2139.
32. Farmer, E.E. and Ryan, C.A., 1990, Interplant communication—airborne methyl jasmonate induces synthesis of proteinase-inhibitors in plant leaves. *Proc. Nat. Acad. Sci. USA*, 87:7713–7716.
33. Feline, T.C., Jones, R.B., Mellows, G., and Phillips, L., 1977, Pseudomonic acid. Part 2. Biosynthesis of pseudomonic acid A. *J. Chem. Soc. Perkin*, 1:309–318.
34. Ferrer, J., Jez, J.M., Bowman, M.E., Dixon, R.A., and Noel, J.P., 1999, Structure of chalcone synthase and the molecular basis of plant polyketide biosynthesis. *Nat. Struct. Biol.*, 6:775–784.
35. Fujii, I., Watanabe, A., Sankawa, U., and Ebizuka, Y., 2001, Identification of Clascin cyclase domain in fungal polyketide synthase WA, a naphthopyrone synthase of *Aspergillus nidulans*. *Chem. Biol.*, 8:189–197.
36. Fuller, A.T., Mellows, G., Woodford, M., Banks, G.T., Barrow, K.D., and Chain, E.B., 1971, Pseudomonic acid: An antibiotic produced by *Pseudomonas fluorescens*. *Nature*, 234:416–417.
37. Funai, N., Ohnishi, Y., Fujii, I., Shibuya, M., Ebizuka, Y., and Horinouchi, S., 1999, A new pathway for polyketide synthesis in microorganisms. *Nature*, 400:897–899.
38. Fuqua, C., Winans, S.C., and Greenberg, E.P., 1996, Census and consensus in bacterial ecosystems: The LuxR-LuxI family of quorum-sensing transcriptional regulators. *Annu. Rev. Microbiol.*, 50:727–751.
39. Gandecha, A.R., Large, S.L., and Cundliffe, E., 1997, Analysis of four tylosin biosynthetic genes from the *tylLM* region of the *Streptomyces fradiae* genome. *Gene*, 184:197–203.
40. Gottesman, S., 1996, Proteases and their targets in *Escherichia coli*. *Annu. Rev. Genet.*, 30:465–506.
41. Greenberg, E.P., 2000, Acyl-homoserine lactone quorum sensing in bacteria. *J. Microbiol.*, 38:117–121.

42. Gundlach, H., Muller, M.J., Kutchan, T.M., and Zenk, M.H., 1992, Jasmonic acid is a signal transducer in elicitor-induced plant-cell cultures. *Proc. Nat. Acad. Sci. USA*, 89:2389–2393.
43. Haas, D., Blumer, C., and Keel, C., 2000, Biocontrol ability of fluorescent pseudomonads genetically dissected: Importance of positive feedback regulation. *Curr. Opin. Biotechnol.*, 11:290–297.
44. Hayashi, T., Matsumoto, H., Ohnishi, M., and Terawaki, Y., 1993, Molecular analysis of a cytotoxin-converting phage ϕ CTX, of *Pseudomonas aeruginosa*: Structure of the *att-cos-ctx* region and integration into serine tRNA gene. *Mol. Microbiol.*, 7:657–667.
45. Hendrickson, L., Davis, C.R., Roach, C., Nguyen, D.K., Aldrich, T., McAda, P.C., and Reeves, C.D., 1999, Lovastatin biosynthesis in *Aspergillus terreus*: Characterization of blocked mutants, enzyme activities and a multifunctional polyketide synthase gene. *Chem. Biol.*, 6:429–439.
46. Howell, C.R. and Stipanovic, R.D., 1980, Suppression of *Pythium ultimum*-induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic, pyoluteorin. *Phytopathology*, 70:712–715.
47. Hughes, J. and Mellows, G., 1978, Inhibition of isoleucyl-transfer ribonucleic acid synthase in *Escherichia coli* by pseudomonic acid. *Biochem. J.*, 176:305–318.
48. Hughes, J. and Mellows, G., 1980, Interaction of pseudomonic acid A with *Escherichia coli* B isoleucyl-tRNA synthase. *Biochem. J.*, 191:209–219.
49. Ichihara, A., Shiraiishi, K., Sato, H., Sakamura, S., Nishiyama, K., Sakai, R., Furusaki, K., and Matsumoto, T., 1977, The structure of coronatine. *J. Am. Chem. Soc.*, 99:636–637.
50. Isnansetyo, A., Horikawa, M., and Kamei, Y., 2001, *In vitro* anti-methicillin-resistant *Staphylococcus aureus* activity of 2,4-diacetylphloroglucinol produced by *Pseudomonas* sp. AMSN isolated from a marine alga. *J. Antimicrob. Chemother.*, 47:719–730.
51. Keel, C., Schnider, U., Maurhofer, M., Voisard, C., Laville, J., Burger, U., Wirthner, P., Haas, D., and Defago, G., 1992, Suppression of root diseases by *Pseudomonas fluorescens* CHAO: Importance of the bacterial secondary metabolite 2,4-diacetylphloroglucinol. *Mol. Plant Microbe Interact.*, 5:4–13.
52. Keel, C., Weller, D.M., Natsch, A., Defago, G., Cook, R.J., and Thomashow, L.S., 1996, Conservation of the 2,4-diacetylphloroglucinol biosynthesis locus among fluorescent *Pseudomonas* strains from diverse geographic locations. *Appl. Environ. Microbiol.*, 62:552–563.
53. Kellner, R.L.L., 2002, Molecular identification of an endosymbiotic bacterium associated with pederin biosynthesis in *Paederus sabaesus* (Coleoptera: Staphylinidae). *Insect Biochem. Mol. Biol.*, 32:389–395.
54. Kellner, R.L.L., 2001, Suppression of pederin biosynthesis through antibiotic elimination of endosymbionts in *Paederus sabaesus*. *J. Insect Physiol.*, 47:475–483.
55. Kellner, R.L.L., 1999, What is the basis of pederin polymorphism in *Paederus riparius* rove beetles? The endosymbiotic hypothesis. *Entomol. Exp. Appl.*, 93:41–49.
56. Kellner, R.L.L. and Dettner, K., 1996, Differential efficacy of toxic pederin in deterring potential arthropod predators of *Paederus* (Coleoptera: Staphylinidae) offspring. *Oecologia*, 107:293–300.
57. Kenyon, J.S. and Turner, J.G., 1992, The stimulation of ethylene synthesis in *Nicotiana tabacum* leaves by the phytotoxin coronatine. *Plant Physiol.*, 100:219–224.
58. Kraus, J. and Loper, J.E., 1995, Characterisation of a genomic region required for production of the antibiotic pyoluteorin by the biological control agent *Pseudomonas fluorescens* Pf-5. *Appl. Environ. Microbiol.*, 61:849–854.
59. Kraus, J. and Loper, J.E., 1992, Lack of evidence for a role of antifungal metabolite production by *Pseudomonas fluorescens* Pf-5 in biological control of phythium damping-off of cucumber. *Phytopathology*, 82:264–271.

60. Levy, E., Gough, F.J., Berlin, K.D., Guiana, P.W., and Smith, J.T., 1992, Inhibition of *Septoria tritici* and other phytopathogenic fungi and bacteria by *Pseudomonas fluorescens* and its antibiotics. *Plant Pathol.*, 41:335–341.
61. Liang, L.Z., Sobiczewski, P., Paterson, J.M., and Jones, A.L., 1994, Variation in virulence, plasmid content, and genes for coronatine synthesis between *Pseudomonas syringae* pv. *morsprunorum* and *P. s. syringae* from *Prunus*. *Plant Dis.*, 78:389–392.
62. Liyanage, H., Palmer, A., Ullrich, M., and Bender, C.L., 1995, Characterization and transcriptional analysis of the gene cluster for coronafacic acid, the polyketide component of the phytotoxin coronatine. *Appl. Environ. Microbiol.*, 61:3843–3848.
63. Liyanage, H., Penfold, C., Turner, J., and Bender, C.L., 1995, Sequence, expression and transcriptional analysis of the coronafacate ligase-encoding gene required for coronatine biosynthesis by *Pseudomonas syringae*. *Gene*, 153:17–23.
64. MacNeil, D.J., Occi, J.L., Gewain, K.M., MacNeil, T., Gibbons, P.H., Ruby, C.L., and Danis, S.J., 1992, Complex organisation of the *Streptomyces avermitilis* genes encoding the avermectin polyketide synthase. *Gene*, 115:119–125.
65. Malpartida, F. and Hopwood, D.A., 1986, Physical and genetic characterisation of the gene cluster for the antibiotic actinorhodin in *Streptomyces coelicolor* A3(2). *Mol. Gen. Genet.*, 205:66–73.
66. Mantle, P.G. and MacGeorge, K.M., 1991, Pseudomonic acid biosynthesis. The putative role of 3-hydroxy-3-methylglutarate. *J. Chem. Soc. Perkin Transact.*, 1:255–258.
67. Martin, F.M. and Simpson, T.J., 1989, Biosynthetic studies on pseudomonic acid (mupirocin), a novel antibiotic metabolite of *Pseudomonas fluorescens*. *J. Chem. Soc. Perkin Transact.*, 1:207–209.
68. Matsumoto, T., Yanagiya, M., Maeno, S., and Yasuda, S., 1968, A revised structure of pederin. *Tetrahedron Lett.*, 60:6297–6300.
69. Maurhofer, M., Keel, C., Haas, D., and Defago, G., 1995, Influence of plant species on disease suppression by *Pseudomonas fluorescens* strain CHAO with enhanced antibiotic production. *Plant Pathol.*, 44:40–50.
70. Maurhofer, M., Keel, C., Haas, D., and Defago, G., 1994, Pyoluteorin production by *Pseudomonas fluorescens* strain CHAO is involved in the suppression of *Pythium* damping-off of cress but not cucumber. *Eur. J. Plant Pathol.*, 100:221–232.
71. Mitchell, R.E., 1991, Implications of toxins in the ecology and evolution of plant pathogenic microorganisms-bacteria. *Experientia*, 47:791–803.
72. Mitchell, R.E. and Frey, E.J., 1986, Production of N-coronafacoyl-L-amino-acid-analogs of coronatine by *Pseudomonas syringae* pv. *atropurpurea* in liquid cultures supplemented with L-amino-acids. *J. Gen. Microbiol.*, 132:1503–1507.
73. Mitchell, R.E., Young, H., and Liddell, M.J., 1995, Isolation and structural characterisation of 2-[1-oxo-2-cyclopenten-2-ylmethyl]-butanoic acid, a polyketide product of coronatine-producing *Pseudomonas* spp. *Tetrahedron Lett.*, 36:3237–3240.
74. Mitchell, R.E., Young, S.A., and Bender, C.L., 1994, Coronamic acid, an intermediate in coronatine biosynthesis by *Pseudomonas syringae*. *Phytochemistry*, 35:343–348.
75. Montamedi, H. and Hutchinson, C.R., 1987, Cloning and heterologous expression of a gene cluster for the biosynthesis of tetracenomycin C, the anthracycline antibiotic of *Streptomyces glaucescens*. *Proc. Nat. Acad. Sci. USA*, 84:4445–4449.
76. Nowak-Thompson, B., Chaney, N., Wing, J.S., Gould, S.J., and Loper, J.E., 1999, Characterization of the pyoluteorin biosynthetic gene cluster of *Pseudomonas fluorescens* Pf-5. *J. Bacteriol.*, 181:2166–2174.
77. Nowak-Thompson, B., Gould, S.J., Kraus, J., and Loper, J.E., 1994, Production of 2,4-diacetylphloroglucinol by the biocontrol agent *Pseudomonas fluorescens* Pf-5. *Can. J. Microbiol.*, 40:1064–1066.

78. Nowak-Thompson, B., Gould, S.J., and Loper, J.E., 1997, Identification and sequence analysis of the genes encoding a polyketide synthase required for pyoluteorin biosynthesis in *Pseudomonas fluorescens* Pf-5. *Gene*, 204:17–24.
79. O'Hanlon, P.J., Rogers, N.H., and Tyler, J.W., 1983, The chemistry of pseudomonic acid. Part 6. Structure and preparation of pseudomonic acid D. *J. Chem. Soc. Perkin Transact.*, 1:2655–2665.
80. Pao, G.M., Tam, R., Lipschitz, L.S., and Saier, M.H., 1994, Response regulators-structure, function and evolution. *Res. Microbiol.*, 145:356–362.
81. Parkinson, J.S. and Kofoed, E.C., 1992, Communicating modules in bacterial signaling proteins. *Annu. Rev. Genet.*, 26:71–112.
82. Parry, R.J., Jiralerspong, S., Mhaskar, S., Alemany, L., and Willcott, R., 1996, Investigations of coronatine biosynthesis. Elucidation of the mode of incorporation of pyruvate into coronafacic acid. *J. Am. Chem. Soc.*, 118:703–704.
83. Parry, R.J., Mhaskar, S.V., Lin, M., Walker, A.E., and Mafoti, R., 1994, Investigations of the biosynthesis of the phytotoxin coronatine. *Can. J. Chem.*, 72:86–99.
84. Penaloza-Vazquez, A. and Bender, C.L., 1998, Characterization of CorR, a transcriptional activator which is required for biosynthesis of the phytotoxin coronatine. *J. Bacteriol.*, 180:6252–6259.
85. Penfold, C.N., Bender, C.L., and Turner, J.G., 1996, Characterisation of genes involved in biosynthesis of coronafacic acid, the polyketide component of the phytotoxin coronatine. *Gene*, 183:167–173.
86. Piel, J., 2002, A polyketide synthase-peptide synthetase gene cluster from an uncultured bacterial symbiont of *Paederus* beetles. *Proc. Nat. Acad. Sci. USA*, 99:14002–14007.
87. Rangaswamy, V., Jiralerspong, S., Parry, R., and Bender, C.L., 1998, Biosynthesis of the *Pseudomonas* polyketide coronafacic acid requires monofunctional and multifunctional polyketide synthase proteins. *Proc. Nat. Acad. Sci. USA*, 95:15469–15474.
88. Rangaswamy, V., Mitchell, R., Ullrich, M., and Bender, C.L., 1998, Analysis of genes involved in biosynthesis of coronafacic acid, the polyketide component of the phytotoxin coronatine. *J. Bacteriol.*, 180:3330–3338.
89. Rangaswamy, V., Ullrich, M., Jones, W., Mitchell, R., Parry, R., Reynolds, P., and Bender, C.L., 1997, Expression and analysis of coronafacate ligase, a thermoregulated gene required for production of the phytotoxin coronatine in *Pseudomonas syringae*. *FEMS Microbiol. Lett.*, 154:65–72.
90. Richter, A., Kocienski, P., Raubo, P., and Davies, D.E., 1997, The in vitro biological activities of synthetic 18-O-methyl mycalamide B, 10-epi-18-O-methyl mycalamide B and pederin. *AntiCancer Drug Des.*, 12:217–227.
91. Rohde, B.H., Pohlack, B., and Ullrich, M.S., 1998, Occurrence of thermoregulation of genes involved in coronatine biosynthesis among various *Pseudomonas syringae* strains. *J. Basic Microbiol.*, 38:41–50.
92. Sarniguet, A., Kraus, J., Henkels, M.D., Muehlchen, A.M., and Loper, J.E., 1995, The sigma factor σ^S affects antibiotic production and biological control activity of *Pseudomonas fluorescens* Pf-5. *Proc. Nat. Acad. Sci. USA*, 92:12255–12259.
93. Schell, M.A., 1993, Molecular biology of the LysR family of transcriptional regulators. *Annu. Rev. Microbiol.*, 47:597–626.
94. Schnider, U., Keel, C., Blumer, C., Troxler, J., Defago, G., and Haas, D., 1995, Amplification of the housekeeping sigma factor in *Pseudomonas fluorescens* CHAO enhances antibiotic production and improves biocontrol abilities. *J. Bacteriol.*, 177:5387–5392.
95. Schnider-Keel, U., Seematter, A., Maurhofer, M., Blumer, C., Duffy, B., Gigot-Bonnefoy, C., Reimann, C., Notz, R., Defago, G., Haas, D., and Keel, C., 2000, Autoinduction of 2,4-diacylphloroglucanone biosynthesis in the biocontrol agent *Pseudomonas fluorescens*

- CHAO and repression by the bacterial metabolites salicylate and pyoluteorin. *J. Bacteriol.*, 182:1215–1225.
96. Schroder, J., 1999, Probing plant polyketide biosynthesis. *Nat. Struct. Biol.*, 6:714–716.
 97. Schwicke, T., Aparicio, J.F., Molnar, I., Konig, A., Khaw, L.E., Haydock, S.F., Oliynyk, M., Caffrey, P., Cortes, J., Lester, J.B., Bohm, G.A., Staunton, J., and Leadlay, P.F., 1995, The biosynthetic gene cluster for the polyketide immunosuppressant rapamycin. *Proc. Nat. Acad. Sci. USA*, 92:7839–7843.
 98. Sembdner, G. and Parthier, B., 1993, The biochemistry and the physiological and molecular actions of jasmonates. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 44:569–589.
 99. Shanahan, P., Glennon, J.D., Crowley, J.J., Donnelly, D.F., and O’Gara, F., 1993, Liquid chromatographic assay of microbially derived phloroglucinol antibiotics for establishing the biosynthetic route to production, and the factors affecting their regulation. *Anal. Chim. Acta*, 272:271–277.
 100. Shanahan, P., O’Sullivan, D.J., Simpson, P., Glennon, J.D., and O’Gara, F., 1992, Isolation of 2,4-diacetylphloroglucinol from a fluorescent pseudomonad and investigation of physiological parameters influencing its production. *Appl. Environ. Microbiol.*, 58:353–358.
 101. Shiozawa, H., Shimada, A., and Takahashi, S., 1997, Thiomarinols D, E, F, and G, new hybrid antimicrobial antibiotics produced by a marine bacterium. Isolation, structure, and antimicrobial activity. *J. Antibiot.*, 50:449–452.
 102. Stachelhaus, T. and Marahiel, M.A., 1995, Modular structure of genes encoding multifunctional peptide synthetases required for non-ribosomal peptide synthesis. *FEMS Microbiol. Lett.*, 125:3–14.
 103. Stutz, E.W., Defago, G., and Kern, H., 1986, Naturally occurring fluorescent Pseudomonads involved in suppression of black root rot of tobacco. *Phytopathology*, 76:181–185.
 104. Stutzman-Engwall, K.J. and Hutchinson, C.R., 1989, Multigene families for anthracycline antibiotic production in *Streptomyces peuceitius*. *Proc. Nat. Acad. Sci. USA*, 86: 3135–3139.
 105. Sugden, M.J., 1992, The biosynthesis of pseudomonic acid. Ph.D. thesis. University of Bristol.
 106. Thomas, M.G., Burkart, M.D., and Walsh, C.T., 2002, Conversion of L-proline to pyrrolyl-2-carboxy-S-PCP during undecylprodigiosin and pyoluteorin biosynthesis. *Chem. Biol.*, 9:171–184.
 107. Tsay, J.T., Oh, W., Larson, T.J., Jackowski, S., and Rock, C.O., 1992, Isolation and characterization of the β -ketoacyl-acyl carrier protein synthase III gene (*fabH*) from *Escherichia coli* K-12. *J. Biol. Chem.*, 267:6807–6814.
 108. Ullrich, M. and Bender, C.L., 1994, The biosynthetic gene cluster for coronamic acid, an ethylcyclopropyl amino acid, contains genes homologous to amino acid-activating enzymes and thioesterases. *J. Bacteriol.*, 176:7574–7586.
 109. Ullrich, M., Guenzi, A.C., Mitchell, R.E., and Bender, C.L., 1994, Cloning and expression of genes required for coronamic acid (2-ethyl-1-aminocyclopropane 1-carboxylic acid), an intermediate in the biosynthesis of the phytotoxin coronatine. *Appl. Environ. Microbiol.*, 60:2890–2897.
 110. Ullrich, M., A. Penaloza-Vazquez, Bailey, A., and Bender, C.L., 1995, A modified two-component regulatory system is involved in temperature-dependent biosynthesis of the *Pseudomonas syringae* phytotoxin coronatine. *J. Bacteriol.*, 177:6160–6169.
 111. van Pee, K.H., 1996, Biosynthesis of halogenated metabolites by bacteria. *Annu. Rev. Microbiol.*, 50:375–399.
 112. Vincent, M.N., Harrison, L.A., Brackin, J.M., Kovacevich, P.A., Mukerji, P., Weller, D.M., and Pierson, E.A., 1991, Genetic analysis of the antifungal activity of a soilborne *Pseudomonas aureofaciens* strain. *Appl. Environ. Microbiol.*, 57:2928–2934.

113. Wang, W., Alarcon-Chaidez, F., A. Penaloza-Vazquez, and Bender, C.L., 2002, Differential regulation of coronatine biosynthesis in *Pseudomonas syringae* pv. *tomato* DC3000 and *P. syringae* pv. *glycinea* PG4180. *Physiol. Mol. Plant Pathol.*, 60:111–120.
114. Weiler, E.W., Kutchan, T.M., Gorba, T., Brodschelm, W., Niesel, U., and Bublitz, F., 1994, The *Pseudomonas* phytotoxin coronatine mimics octadecanoid signalling molecules of higher plants. *FEBS Lett.*, 345:9–13.
115. Whatling, C.A., Hodgson, J.E., Burnham, M.K., R., Clarke, N.J., Franklin, F.C.H., and Thomas, C.M., 1995, Identification of a 60 kb region of the chromosome of *Pseudomonas fluorescens* NCIB 10586 required for the biosynthesis of pseudomonic acid (mupirocin). *Microbiol.*, 141:973–982.
116. Whistler, C.A., Corbell, N.A., Sarniguet, A., Ream, W., and Loper, J.E., 1998, The two-component regulators GacS and GacA influence accumulation of the stationary-phase sigma factor σ^S and the stress response in *Pseudomonas fluorescens* Pf-5. *J. Bacteriol.*, 180:6635–6641.
117. Whistler, C.A., Stockwell, V.O., and Loper, J.E., 2000, Lon protease influences antibiotic production and UV tolerance of *Pseudomonas fluorescens* Pf-5. *Appl. Environ. Microbiol.*, 66:2718–2725.
118. Yanagisawa, T., Lee, J.T., Wu, H.C., and Kawakami, M., 1994, Relationship of protein-structure of isoleucyl-transfer-RNA synthetase with pseudomonic acid resistance of *Escherichia coli* - proposed mode of action of pseudomonic acid as an inhibitor of isoleucyl-transfer-RNA synthetase. *J. Biol. Chem.*, 269:24304–24309.
119. Young, S.A., Park, S.K., Rodgers, C., Mitchell, R.E., and Bender, C.L., 1992, Physical and functional characterisation of the gene cluster encoding the polyketide phytotoxin coronatine in *Pseudomonas syringae* pv. *glycinea*. *J. Bacteriol.*, 174:1837–1843.
120. Yuan, Z., Cang, S., Matsufuji, M., Nakata, K., Nagamatsu, Y., and Yoshimoto, A., 1998, High production of pyoluteorin and 2,4-diacetylphloroglucinol by *Pseudomonas fluorescens* S272 grown on ethanol as a sole carbon source. *J. Ferment. Bioeng.*, 86:559–563.

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